

Universidade de Lisboa
Faculdade de Ciências
Departamento de Física



Adipose Stromal Vascular Fraction Cell Sheets as Vascular Strategies for
Tissue Engineering and Regenerative Medicine Applications

Marina Filipa da Silva Costa

Dissertação orientada por:

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Mestrado Integrado em Engenharia Biomédica e Biofísica
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Abbreviations

α - MEM - Minimum Essential Medium with Alpha Modifications

ANG – Angiopoietin

ABTS - 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)

APS - Ammonium Persulfate

ARNT - Aryl Hydrocarbon Receptor Nuclear Translocator

BMSCs – Bone Marrow Stem Cells

BSA – Bovine Serum Albumin

CS – Cell Sheet(s)

CSE – Cell Sheet Engineering

DNA - Deoxyribonucleic acid

dsDNA – Double-stranded DNA

ECs – Endothelial Cell(s)

EPC(s) – Endothelial Progenitor Cell(s)

ELISA – Enzyme-linked Immunosorbent Assay

ECM – Extracellular Matrix

FGF - Fibroblast Growth factor

FBS – Fetal Bovine Serum

hASCs – Human Adipose Stem Cells

HIF – Hypoxia inducible factor

MSC – Mesenchymal Stem Cells

ODD – Oxygen-Dependent Degradation Domain

PBS – Phosphate Buffered Saline

PDGF-BB – Platelet-derived Growth Factor subunit B

PNIPAM - poly(*N*-isopropylacrylamide)

RM – Regenerative Medicine

SVF – Stromal Vascular Fraction

TEMED - Tetramethylethylenediamine

TE – Tissue Engineering

TERM – Tissue Engineering and Regenerative Medicine

TIMP1 – Tissue Inhibitor of Metalloproteinases

Te - Tris-EDTA (Ethylenediamine Tetraacetic Acid; buffered solution)

TGF – Transforming Growth Factor

TNF alpha – Tumor Necrosis Factor Alpha

VEGF – Vascular Endothelial Growth Factor

VHL – Von Hippel Lindau

ABSTRACT

Tissue Engineering (TE), a subfield of Regenerative Medicine, arises as an attempt to repair diseases or defects by using cells, tissues and biomaterials isolated or in combination, to restore or improve biological functions. Cell sheet (CS) engineering is a TE cell-based technique that uses stimuli-responsive culture surfaces to fabricate robust and intact cell monolayers that can be implanted without requiring the support of a scaffold. The creation of viable thick CS constructs is limited by the lack of suitable vascularization strategies prior to implantation. The herein described strategy results from the optimization of different aspects of cell culture to obtain cell sheets capable of developing a capillary-like network *in vitro* without adding extrinsic growth factors. These cell sheets are derived from the isolation of the stromal vascular fraction (SVF) of adipose tissue, which is composed of different cell populations including stromal, endothelial and hematopoietic cells, holding thus an intrinsic angiogenic potential. The culture was performed with basal medium in normoxic ($pO_2 = 21\%$) and hypoxic ($pO_2 = 5\%$) conditions for up to 8 days. Cell proliferation results showed no significant differences between all conditions. Immunocytochemistry performed with the endothelial marker CD 31 and CD 146 that stains pericytes demonstrated the organization of SVF cells in capillary-like structures for both conditions, although the level of branching was much more complex in hypoxia. It was hypothesized that the secretion of growth factors by cells was involved in this effect. A matrigel assay using conditioned media from the different conditions showed increased formation of capillary-like structures by endothelial cells cultured in hypoxic conditioned media. The same conditioned media increased the migration of human adipose stem cells (hASCs) in a scratch assay, when compared with media collected from normoxia. The production of growth factors assessed through ELISA suggested higher secretion of VEGF for hypoxia although statistical analysis showed no significant differences by comparison with normoxia. Western blot for HIF-1 α expression highlighted more prominent bands for hypoxic conditions. In vivo implantation of cell sheets cultured for 8 days in normoxia and hypoxia following a hind limb ischemia model showed improved neo-angiogenesis as evidenced by immunohistochemistry and laser Doppler. These results highlight the outstanding potential of combining hypoxic conditions and SVF cells to create cell sheets that can be used as functional vascularization units for TE and RM.

Keywords: vascularization, SVF, hypoxia, ischemia, regenerative medicine.

RESUMO

A Engenharia de Tecidos (TE), uma subárea da Medicina Regenerativa (RM), surge como uma tentativa de reparar doenças ou defeitos usando células, tecidos e biomateriais, isoladamente ou em combinação, para restaurar ou melhorar funções biológicas. A Engenharia de *Cell Sheet* (CS) é uma técnica de TE baseada em células que usa superfícies de cultura sensíveis a estímulos de temperatura para fabricar monocamadas celulares intactas e robustas que podem ser implantadas sem requerer o apoio de um *scaffold*, evitando assim algumas das várias restrições às quais eles estão associados. A criação de estruturas de CS viáveis e espessas, necessárias para regenerar tecidos mais robustos como o osso, é limitada pela falta de estratégias de vascularização eficazes, antes da implantação. A falta de vascularização adequada resulta, muitas vezes, em necrose celular e rejeição da estrutura sendo, por isso, uma das maiores preocupações na área da TE. Na comunidade científica, diversas estratégias foram propostas neste sentido, muitas delas envolvendo o uso de células endoteliais que, embora bastante promissoras, enfrentam múltiplos desafios como sendo as fontes de células e a estabilidade dos novos vasos sanguíneos formados. A estratégia aqui descrita resulta de uma otimização dos diferentes aspectos relativos à cultura celular para obter CS capazes de desenvolver, *in vitro*, uma rede de estruturas semelhantes a capilares sem adição de factores de crescimento extrínsecos. Estas CS são derivadas do isolamento da fracção vascular estromal (SVF) do tecido adiposo após dissociação enzimática por acção da collagenase. Este tecido tem vindo a destacar-se na comunidade científica pelas suas características: é abundante, prescindível e pode ser facilmente obtido. Para além disso, a SVF é considerada como sendo detentora de um potencial angiogénico intrínseco, em virtude do tipo de células que a compõem. As células da SVF isoladas a partir de tecido proveniente de lipoaspirações foram distribuídas por placas estéreis de 24 poços numa densidade de 2×10^5 células nucleadas/poço e cultivadas em condições de normóxia (21%) e hipóxia (5% de oxigénio) com meio basal durante 8 dias. Foram efectuados diversos testes *in vitro* após terem sido atingidos os *time points* de 5 e 8 dias, nomeadamente citometria de fluxo (também efectuada imediatamente após o isolamento), quantificação de ADN, ensaios de matrigel e *scratch*, ELISA, imunocitoquímica e *western blot*. Para além disso, foram também efectuados ensaios *in vivo* após indução de isquemia nos membros posteriores que foram avaliados por imunohistoquímica, hibridização *in situ* e *laser Doppler*.

A análise dos marcadores de superfície das células da SVF por citometria de fluxo evidenciou que a expressão dos marcadores endotelial CD 31 e hematopoiético CD 34 decresce ao longo do tempo, o que seria expectável uma vez que a cultura foi efectuada em meio basal, sem adição de factores de crescimento extrínsecos. Na literatura, a SVF do tecido adiposo é descrita como

sendo composta por diversas populações de células, incluindo células estromais, endoteliais e hematopoiéticas, facto que foi comprovado nesta análise. Os resultados relativos à proliferação celular de células da SVF mostram que não há diferenças significativas entre as condições de normóxia e hipóxia sugerindo que a proliferação destas células não é afetada significativamente pelas concentrações de oxigénio. A imunocitoquímica realizada para os marcadores CD 31 e para o CD 146, o qual marca péricitos, demonstram a organização de células da SVF em estruturas tipo capilares para ambas as condições, embora o nível de ramificação tenha sido mais complexo em hipóxia para 8 dias. A comprovar este facto, a quantificação das estruturas tipo capilares no *Angiogenesis Analyzer*, um *plug-in* do programa Image J, destacou que as formações seriam mais maduras e organizadas nessas mesmas condições. Esse facto tornou-se mais evidente num dos dados devido ao decréscimo no número de segmentos e malhas formados pelas células ao longo do tempo, sendo que o nível de ramificação se manteve praticamente inalterado. Isso traduziu-se em segmentos mais alongados e interconectados entre si. Foi levantada a hipótese de que a secreção de factores de crescimento pelas células em condições de hipóxia poderia estar envolvida neste efeito. Como tal, foram efectuados diversos ensaios utilizando os meios condicionados recolhidos das culturas em diferentes condições. Um ensaio de matrigel utilizando um extracto de membrana basal proteico que favorece a angiogénese, evidenciou um aumento na formação de estruturas tipo capilares pelas células endoteliais cultivadas em meio condicionado hipóxico. Os mesmos meios condicionados aumentaram a migração de células estaminais do tecido adiposo humano (hASCs) num ensaio *scratch*, quando comparados com os meios recolhidos de normóxia. O ensaio *scratch* é um ensaio baseado na raspagem da zona central de uma monocamada celular para avaliar a migração das células em torno da área erodida, sob diferentes condições. A produção de factores de crescimento avaliada por meio de ELISA sugeriu uma maior secreção do VEGF para condições de hipóxia. Colocou-se a hipótese que este facto poderia estar relacionado com a estabilização das subunidades dos HIFs (*hypoxia inducible factors*) em condições de concentrações baixas de oxigénio. OS HIFs são uma família de factores de transcrição constituída por três subunidades principais HIF-1, HIF-2 e HIF-3 e os seus respectivos heterodímeros (alfa e beta). As subunidades alfa respondem a variações na concentração de oxigénio e sob condições de oxigénio atmosféricas (normóxia) são constantemente expressas e degradadas no citoplasma, mais concretamente no proteassoma. Em condições de *stress* de oxigénio estas subunidades migram para o núcleo onde se ligam aos seus heterodímeros, os quais são constitutivamente expressos, e estabilizam. Esta estabilização resulta num aumento da secreção de diversos factores de crescimento responsáveis pela regulação do metabolismo da glucose, eritropoiese e angiogénese como sendo o VEGF e o PDGF. Deste modo, foi efectuada uma análise por *western blot* para o HIF-1 α a qual destacou bandas mais proeminentes para condições de hipóxia. Paralelamente às condições de hipóxia e normóxia foram também

testadas a incubação das células com um inibidor da subunidade HIF-1 α e com cloreto de cobalto II, um indutor químico de condições de hipóxia. Como seria expectável, a banda obtida na análise de *western blot* para o inibidor foi bastante ténue contrariamente à obtida com o mimetizador das condições de hipóxia. Em imunocitoquímica isto reflectiu-se num fraco e proeminente desenvolvimento das estruturas tipo capilares, respectivamente, facto que veio reforçar a hipótese proposta.

Finalmente, depois da manutenção das CS por 8 dias em condições de normóxia e hipóxia foi efectuada a sua implantação *in vivo* em ratinhos durante 33 dias, após indução de isquemia no membro posterior. A imunohistoquímica dos explantes recuperados executada para os marcadores endotelial CD 31 e perivascular α -SMA destacou uma melhoria na neo-angiogénese em condições de hipóxia por comparação com as de normóxia e controlos. Foi efectuado também um ensaio de hibridização *in situ* para localizar as células humanas nos explantes e verificou-se que algumas destas células permaneciam na área da implantação em torno dos novos vasos sanguíneos formados. Os resultados obtidos através do *laser Doppler* demonstram que a perfusão dos vasos sanguíneos aumentou nos animais em que foram transplantadas as *cell sheets*. Para além disso, foi observado que nos animais que receberam as *cell sheets* provenientes de culturas em hipóxia havia uma tendência para uma recuperação mais rápida do fluxo sanguíneo. Tendo em consideração todos os resultados obtidos é importante salientar o excelente potencial da estratégia aqui descrita que resulta da combinação de diversos conceitos como sendo, condições de hipóxia, células da SVF e Engenharia de *Cell Sheet* (CS) para criar unidades de vascularização funcionais para TE e RM.

Palavras-Chave: vascularização, SVF, hipóxia, isquemia, medicina regenerativa.

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STRUCTURE

The herein presented thesis is divided in four main chapters: chapter I, the state of the art of the theme; chapter II, the detailed description of all materials and techniques applied throughout the work; chapter III, the scientific paper written during this dissertation project that integrates all the results, discussion and final conclusions which, at the time of the delivery of this thesis was in process of submission, and finally chapter IV, the appendices.

INTRODUCTION

1. Tissue Engineering and Regenerative Medicine

Tissue Engineering (TE) is a forefront area in Health Sciences whose purpose is to rebuild or repair damaged or aged cells, tissues or even entire organs by isolated or combined action of cells, growth factors and biomaterials. It is integrated in the field of Regenerative Medicine (RM) that aims the regeneration of tissues and organs using not only TE but also the potential of genetics and cell therapy. Cell-based approaches such as TE and cell therapy take advantage of the biological properties of mesenchymal stem/stromal cells, which can be found in almost all tissues of the human body, to regenerate tissues or entire organs by transplantation into the injured site. Most of TE strategies require a well-orchestrated sequence of events that range from the extraction of cells, which can be expanded or induced to differentiate upon administration of proper biochemical cues in cell culture surfaces, to its transplantation integrated in 3D scaffolds made of both natural and synthetic biomaterials, in the same patient. In order to be functional after implanted *in vivo*, these scaffolds should have some basic properties: they have to be biocompatible, bioresorbable with a well-controlled degradation rate and facilitate the diffusion of nutrients and oxygen through a porous surface. Besides, this surface should be compatible with cell attachment and constructs must possess specific mechanical properties taking into account the target-tissue[1]. However, biomaterials-based strategies face several constraints associated mainly with immunogenicity problems and poor degradation rate of the scaffold that can lead to fibrosis and necrosis in the bulk of thicker constructs, aiming to target thicker tissues and/or organs[2].

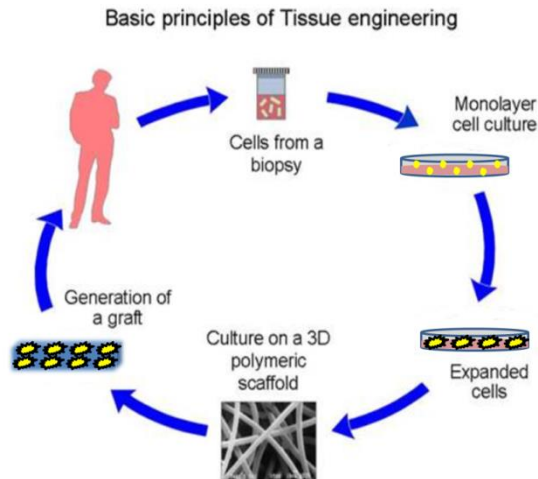


Figure 51- Traditional cell-based approach in TE. Adapted from:
http://www.wikilectures.eu/index.php/Tissue_engineering_principle

Another constraint typically associated with cell-based strategies is cell sourcing. Mesenchymal stem cells, also known as multipotent stromal cells, captivated an overwhelming attention in the scientific world as powerful therapeutic tools and inspired several works, a great part of them using MSCs isolated from the bone marrow. The first pioneer works reporting the discovery of a MSC's niche date back to 1968 when Friedenstein and colleagues discovered that stem cells, derived from the bone marrow (BMSCs), were capable of differentiating into bone [3]. However, the extraction of cells from the bone marrow is often associated with donor-site morbidity, pain and poor yield of cells. Necessity of an alternative spurred scientists to find new MSC sources and currently it is well known that they can be found in almost all tissues of the human body, namely skeletal muscle [4], adipose tissue [5], skin [6], pancreas [7], synovial membrane [8], dental pulp [9], placenta[10], trachea [11], nasal mucosa[12], Wharton's jelly [13] and also amniotic fluid[14], menstrual blood[15], among others.

There are a lot of studies in clinical trials regarding cell-based strategies involving mainly the use of stem cells isolated from bone marrow, adipose tissue, placenta and umbilical cord blood for several different applications. Amongst this wide range of applications it's possible to find immunological and blood diseases, cardiac repair and neurological disorders[16].

2. The problem of Vascularization after implantation

Blood vessels, major components of the circulatory system, are essential for the survival of the tissues and organs by supplying the nutrients and oxygen and collecting the waste products from cellular metabolism, regulating thus diverse functions that allow maintaining homeostasis. Fast

neovascularization is a critical aspect for the effective survival of tissue engineered constructs in order to avoid hypoxia and/or anoxia in the inner mass of tissue-engineered constructs, due to a poor diffusion of nutrients and oxygen essential for their successful engraftment. Ultimately, this poor supply of nutrients and oxygen can culminate in unsatisfactory tissue regeneration or even, failure of the construct. Nonetheless, is difficult to build whole prevascularized implants that could mimic tissues and organs thickness without compromising the distance between cells and blood vessels.

Neovascularization comprises three distinct processes: vasculogenesis, angiogenesis and arteriogenesis. Vasculogenesis corresponds to the process by which *de novo* vessels are formed during the embryonic phase by endothelial progenitors[17] developing a primitive vascular plexus. The sprouting and stabilization processes that follow vasculogenesis and the reshuffle of the early vasculature into a complex and functional circuit are called angiogenesis and arteriogenesis. For that purpose, the secretion of diverse angiogenic growth factors and recruitment of mural cells like smooth muscle cells and pericytes are required to promote ECs survival, proliferation and differentiation as well as the complexity of the blood vessels[18]. Vascular endothelial growth factor (VEGF), angiopoietin (ANG), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), TIMP (tissue inhibitor of metalloproteinases), transforming growth factor (TGF- α) and (TGF- β), hepatocyte growth factor (HGF), tumor necrosis factor- α (TNF- α), proteins, cytokines are also known for playing an active role in the process of neovascularization[17]. VEGF is crucial for modulating endothelial progenitor cells (EPCs) behavior [19] and endothelial cells (ECs) survival, inducing a powerful angiogenic response for both neovascularization and pathological conditions[20]. Moreover, stabilization and maturation of primary capillary plexus depends on the coordinated action of PDGF, TIMP (by remodeling of the ECM during the sprouting), TGF- β and FGF (angiogenic promoters released from the ECM by proteinases action)[17].

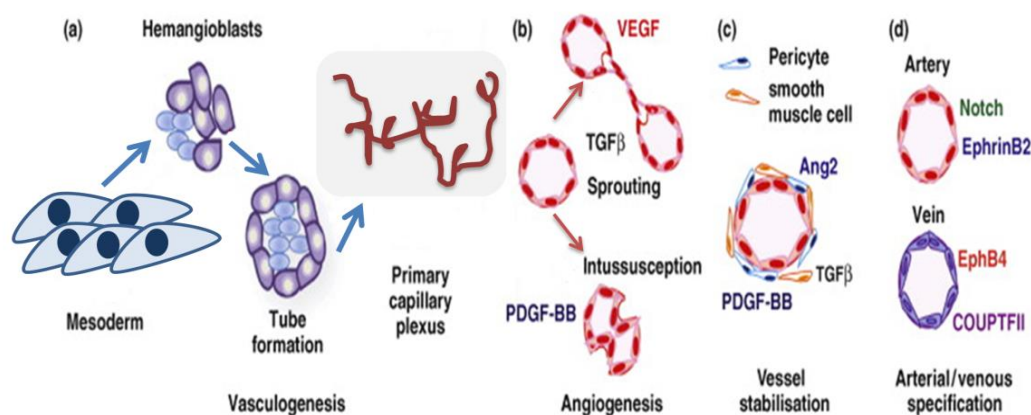


Figure 62 - Formation of blood vessels is regulated by three fundamental processes: vasculogenesis (during the embryo development), angiogenesis and arteriogenesis. Adapted from [21].

Several strategies mainly related with *in vitro* and *in vivo* prevascularized engineered constructs, which can involve angiogenic factors or scaffolds, are under investigation, many of them reporting the use of endothelial cells [22–25]. For instance, transplantation of *ex vivo* expanded human endothelial cells was found to increase the blood vessels and capillary perfusion in a hind limb ischemia induced in mice by Kalka et. al[23]. The same type of cells expanded also *ex vivo* were used as a therapeutic tool in myocardial ischemia[25]. Co-culturing endothelial cells with bone marrow derived stem cells promoted vascularization after implantation[26,27]. Nevertheless, the previously described strategies reported fairly late blood vessels formation. Scaffolds have been also widely used to support the formation of blood vessels. For instance Santos et. al engineered a cell-based construct using a blend of starch with polycaprolactone and co-cultures of endothelial cells and osteoblasts for bone tissue regeneration. The culture in the first 21 days lead to the formation of microcapillary-like structures, more branched and developed after 35 days[28]. Osteogenic/vasculogenic constructs based on porous hydroxyapatite scaffolds and SVF cells cultured *in vitro* for 5 days were found to form blood vessels and bone tissue 8 weeks post-transplantation in nude mice[29]. Moreover, spheroid co-cultures of endothelial and osteoprogenitor cells resulted in the formation of prevascularized networks of capillary-like structures 10 days after *in vitro* culture for bone tissue engineering applications. However, integration with host vasculature after implantation of the construct was found to be poor[30]. The controlled release of angiogenic factors, in the case bFGF, has been achieved by incorporating microspheres composed of poly (lactic-co-glycolic acid) on alginate scaffolds. Capillary density increased since day 10 until 21 days post-transplantation in rat

peritoneum[31]. Nano-sustained controlled release of VEGF on polylactic acid microspheres in combination with SVF cells increased also capillary density in nude mice, 2 months after implantation[32]. The potential of SVF cells in a process of tissue expansion in rats was found to be facilitated, possibly due to the secretion of EGF, VEGF and bFGF which increased not only blood vessels formation but also cells proliferation[33]. Recently, SVF cells were used as vascularization tools in the generation of a human liver. Co-culturing SVF cells with HepG2 cells resulted in the integration of the first with the host vasculature and in an interactive role with parenchymal cells[34]. Endothelial cells were also used to promote vascularization for regeneration of other tissues such as skin[35] or skeletal muscle[36]. Nevertheless, most of the described strategies have drawbacks such as the late formation of blood vessels in vitro and/or post-implantation, the complexity associated with cultures of more than one cell type isolated from different tissues and formation of fibrous tissue after implantation of engineered constructs. With the exponential growth of the engraftment technology over time there is an increasing need to find more, faster and efficient techniques for neovascularization.

3. Stromal Vascular Fraction derived cells

Adipose tissue was neglected in previous years in the scientific world because it was seen as an organ exclusively associated with the storage and release of lipids. Nonetheless, their unique properties (accessibility, low-cost and abundance) combined with the fact of being a reservoir of stem and stromal cells made it quite captivating as a cell source [5]. Also, as fat tissue lines and protects several parts of the human body it can be easily identified, namely at the intra-abdominal area and subcutaneously, and extracted through minimally invasive procedures.

As stated by the International Society for Cellular Therapy the definition of multipotent mesenchymal stromal cells, typically named mesenchymal stem cells, can be divided in three fundamental topics: the presence or absence of selected surface markers (they must express specific cell surface antigens such as CD73, CD90, and CD105, and lack expression of other markers including CD45, CD34, CD14, CD11b, CD79 α , or CD19 and HLA-DR surface molecules) and adhesion and multipotency in vitro (MSCs must be adherent to plastic when kept under standard culture conditions and have the capacity to differentiate in vitro into osteoblasts, adipocytes, and chondroblasts) [37]. Besides these three fundamental topics, mesenchymal stem cells can also be easily identified by their functional properties: the self-renewal and proliferative capacities and their undifferentiated state.

The initial works regarding the isolation of stem and stromal cells from the adipose tissue were developed in the 60s by Rodbell et al. in rat fat pads. Briefly, Rodbell et al. chopped the fat and then washed it several times in order to remove unwanted cells. The extracellular matrix (ECM) of the remaining tissue was then digested with collagenase and centrifuged for deposition of stromal vascular fraction (SVF) cells. Then, SVF cells were removed by aspiration and fat cells washed. This process of isolation is very similar to the one that is used now, with human lipoaspirate tissues with the addition of a red blood cells lysis buffer to the equation. Nevertheless, literature already describes non-enzymatic methods for SVF isolation [38].

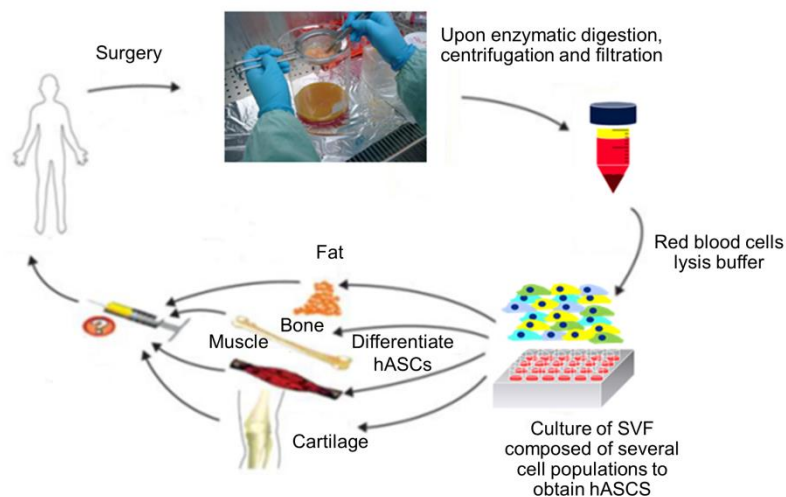


Figure 73 - Isolation and potential applications of human adipose tissue derived stem cells. Adapted from [39].

Adipose-derived stem and stromal cells are multi and pluripotent cells, respectively, that result from the processing of fat tissue. Cells from the stroma of adipose tissue are quite heterogeneous among themselves since they constitute a cocktail of different populations such as pre-adipocytes, mesenchymal progenitors, fibroblasts, pericytes endothelial and hematopoietic cells [40–42]. This heterogeneous composition makes them ideal candidates for a wide range of applications such as neovascularization.

3. The intrinsic angiogenic potential of SVF derived cells

Stromal vascular fraction from adipose tissue has been recently regarded as highly angiogenic and, therefore, quite captivating for improvement of the neovascularization. These cells are known to promote the formation of capillary-like structures both *in vitro* and after transplantation.

Besides its hematopoietic and endothelial composition others factors have been pointed as conditioning for the angiogenic potential of SVF cells. Koh et al. hypothesized that the network of capillary-like structures they are capable to build is created by the reassembly of endothelial progenitors and mural cells contained in the SVF mixture [43]. Dong et Al. also hypothesized that the recruitment of M2 macrophages may also constitute one important factor influencing this angiogenic potential of SVF [44]. All of the research involving its angiogenic capacity made SVF cells quite attractive not only for *in vitro* but also for *in vivo* tests. For instance, the transplantation of the stromal vascular fraction from adipose tissue following a murine hind limb ischemia model have also been shown to promote neovascularization [45,46]. The transplantation was also successful and resulted in the same angiogenic effects in random skin flaps[47]. The great ability of these cells to promote angiogenesis has contributed to physiological and pathological wound healing in mice. The same work also highlighted the potential ability of these cells to enhance reepithelialization[48]. A 3D implant using freshly isolated SVF cells was also built for generation of a functional liver tissue [34]. Nevertheless, more studies need to be developed regarding the process by which SVF cells assemble as capillary-like structures and all variables involved in the process.

4. Cell Sheet Technology

To overcome some of the problems arising from previous cell-based approaches, namely the low anchorage of cells at the injured site [49] and the limitations related to the use of biomaterials as scaffolds [50], the concept of Cell Sheet Engineering was proposed in 1993, bringing a new approach to tissue engineering: the recovery of a contiguous cell layer as a sheet maintaining the native cell-cell and cell-ECM interactions[51].

4.1 The principle for cell sheet detachment

The basic idea of Prof. Okano's group was to develop a scaffold-free approach by coating culture surfaces through covalent grafting with a temperature-responsive polymer - poly(*N*-isopropyl acrylamide) (PNIPAM) that transits from a dehydrated to a hydrated state at temperatures below 32° C. Thus, PNIPAM is regarded as being hydrophobic at temperatures above 32° C and hydrophilic below these. The group successfully cultured bovine endothelial cells and rat hepatocytes at 37° C until achieving confluence and then, by decreasing the temperature until approximately 20°C, the interactions with the ECM were diminished. The critical value of temperature allowed a spontaneous cell detachment [51].

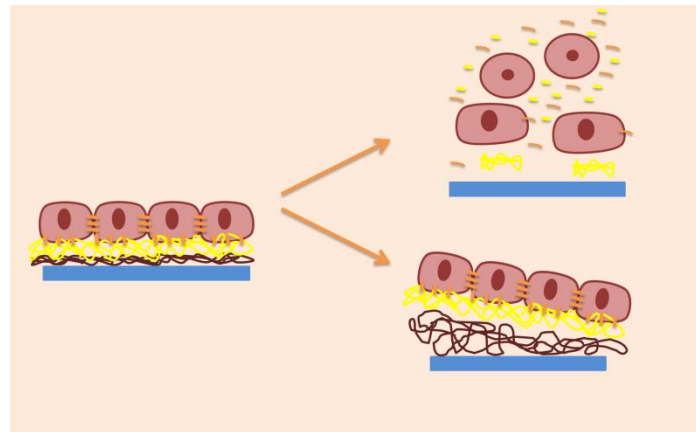


Figure 84 - Cells recovery. Cells can be recovered as single cell suspensions by using proteolytic enzymes that degrade the cell-cell and cell-matrix interactions (upper right) or as cells sheets prepared using a temperature-responsive polymer covalently grafted to a culture dish surface (lower right).

This came to revolutionize the field, as cell sheet engineering (CSE) avoids the use of proteolytic enzymes such as trypsin and dispase, and the native ECM acts as a natural scaffold [52]. Nevertheless, besides these enzymatic treatments and declines of temperature, several other stimuli have been used for cell sheet detachment. Guillaume-Gentil et. al described a technique to obtain viable cell sheets composed of human placenta-derived mesenchymal stem cells based on a electrochemical induction of pH decrease [53]. The same group also described a technique based on polyelectrolyte thin films [54]. Many works using a wide range of different cell types described cell sheet detachments by using magnetic stimuli [55–60]. Through alternation of layer-by-layer depositions of polyanions and polycations, Zahn et. al described an ion-induced cell sheet detachment technique using myoblasts [61]. Micropatterning of the cell culture dishes has been also subject of interest recently and is present as a potential future

strategy [62–64]. This technique takes advantage of the critical temperatures of two different polymers for specific cell adhesion.

4.2 Applications in the Regenerative Field

The potential of cell sheet engineering has been extensively explored over the past few years and now this technology is proposed to improve and treat functions of many tissues such as periodontium [65–67], corneal epithelial [68,69], hepatic [70], urothelial [71,72] and renal [73] tissues, myocardium [74–79], thyroid gland [80], retinal pigment epithelium [81], lung [82,83] and pancreatic islet [84,85]. Besides, cell sheet engineering has been also proposed for esophagus [86–88] and cartilage tissues [89–91]. Our lab, has proposed also the use of CSE to engineer tissues such as skin [92] and bone [93].

Cell sheet engineering has been applied also to improve neovascularization. Asawaka et. al have shown that by stacking human umbilical vein endothelial cells and normal human dermal fibroblasts is possible to build 3D stratified tissues to promote a pre-vascular network both in *vitro* and *in vivo* [94]. Co-cultures involving endothelial cells resulted in increased neovascularization potential and improved cardiac function on ischemic hearts by using tissue-engineered cardiomyocyte sheets [95]. Moreover, monolayered mesenchymal stem cell sheets were found to promote angiogenesis by paracrine pathways on infarcted myocardium [96]. Recent studies have also demonstrated the great ability of this technique to build functional vascularized 3D tissues for cardiac tissue [97].

Cell sheet engineering is also on clinical trials for myocardium - through transplantation of 3-week-cultivated autologous myoblast cell sheets into patients[98] -, cornea - which commonly presents great outcomes[68] -, esophagus[99], periodontium - by using periodontal ligament cell sheets[66] - and cartilage tissues[100]. Nevertheless, literature does not describe yet the use of cell sheet engineering in combination with the whole SVF of adipose tissue.

5. Hypoxia the “physiological normoxia”

Oxygen is a critical component regarding stem and stromal cells culture that is quite often overlooked in the lab context. Since the very beginning of life, the irregularities experienced regarding the oxygen atmospheric concentrations have been boosting the evolution of eukaryotic species in an oxygen-dependent manner. It is believed that there must be a balance between the cellular need for O₂ as a final acceptor of electrons during Krebs cycle and the production of ROS (reactive oxygen species) which lead to oxidative damage and can even cause cell death. This balance is achieved by controlling the O₂ concentrations within the tissues

[101] which are known for being much lower than the regular 21% O₂ atmospheric levels. These lower oxygen levels, in comparison with the atmospheric ones, are called hypoxia. However, as

in vivo physiological oxygen levels are considerably lower than the atmospheric values as cells reside in hypoxic niches this low oxygen tensions are often referenced as “normoxic *in situ*”[101].

5.1 Stem Cells Niche

The stem cell niche can be defined as the microenvironment where stem cells reside. This microenvironment is composed of several components that regulate and control the stem cells fate by sustaining their needs and giving strong cues to direct their activity.

Since the very beginning, during the embryonic phase, O₂ concentrations are constantly varying from the 1-2% when the blastocyst is being implanted [102], rising to the 2-3% with a fully-developed circulatory system and achieving the regular O₂ values within the tissues at the 12-13th week of gestation [103].

In adults, O₂ concentrations within the tissues are dependent on the surrounding blood vessels vascularization but they are considerably lower than the ambient O₂ concentration. Oxygen values in the circulation typically range from 4% to 14% [104], similar to what happens in other organs such as liver [101,105], kidneys [101,106] and lung parenchima [101,107]. In the brain, the oxygen concentrations can be so low as 0.5% being capable to go up until 8% [108]. The oxygen levels in the eye [109] and in the bone marrow [110] range from 1 to 5 and 6%, respectively. In the adipose tissue these concentrations are found to be typically between 2 and 8% [111].

5.2 Hypoxia Inducible Factors: the signaling cascade

Hypoxia-inducible factors (HIFs) are a family of transcription factors responsible for regulating homeostasis, by controlling cellular responses, under low oxygen tensions. The HIF family is composed by three fundamental subunits, HIF-1, HIF-2, and HIF-3, and its corresponding heterodimers (alpha and beta). Alpha subunits are oxygen responsive and become stable in low oxygen conditions by binding their corresponding beta subunits in the nucleus or even in the presence of iron chelators [112]. At regular oxygen conditions, the so called normoxia, these alpha subunits are continuously expressed and degraded promptly. On the other hand, the beta

subunits, also known as aryl hydrocarbon receptor nuclear translocators (ARNTs), are constitutively expressed in both conditions.

HIFs are known for playing a critical role in a wide range of pathological conditions with a special focus in cancer and ischemic diseases. There is evidence in the literature that many tumors propel strongly hypoxic environments that correlate with the progression of the disease by promoting cell invasion and proliferation, angiogenesis and metastasis [113]. This might be due to the fact that HIFs expression triggers the induction of several crucial genes for glycolysis (glucose transporters and glycolytic enzymes) and formation and maturation of the blood vessels such as VEGF, erythropoietin, angiopoietin and platelet-derived growth factor. The latter also highlights why HIFs can be so promising for preventing or even treating ischemic affected areas [113]. For that, understanding HIFs pathway and the role that each specific subunit plays in oxygen deprived conditions is currently the subject of several studies. By now, it is well accepted that all subunits share diverse similarities especially regarding HIF-1 α and HIF-2 α , while HIF-3 α is thought to have an opposite effect under hypoxic conditions. The basic mechanism in the signaling pathway of HIF-1 and HIF-2 at regular O₂ concentrations involves prolyl-hydroxylases that enzymatically induce HIF alpha subunits to bind to von Hippel Lindau (VHL) protein by altering their conformation. The action of the prolyl-hydroxylases occurs in different prolines according to the subunit, hydroxylating them in their respective oxygen-dependent degradation domains (ODD). The lack of O₂ in hypoxic conditions leads to an accumulation of the alpha subunits that translocate to the nucleus, where they bind to their respective ARNT subunit. The transcription of several genes is then activated through the bind of the formed heterodimers to the hypoxia-response elements (HRE) [114–116].

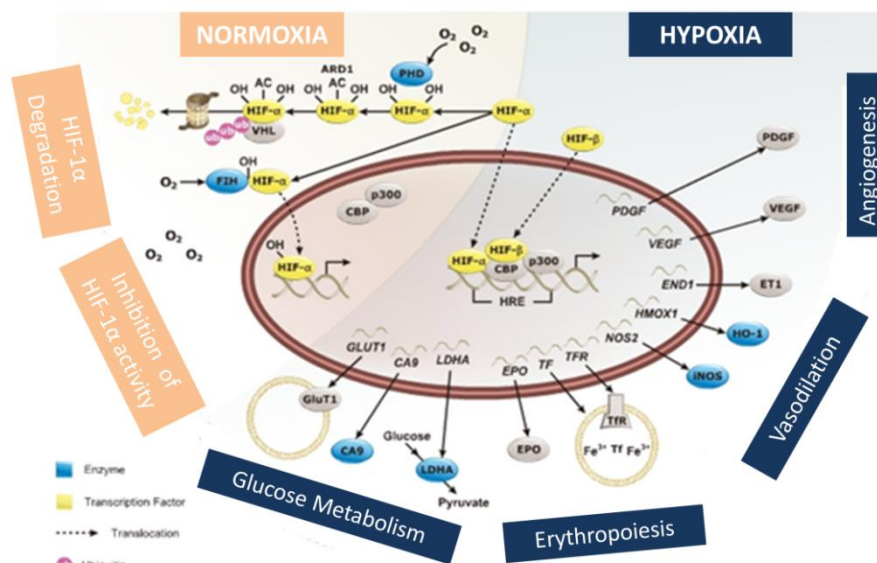


Figure 95 - HIF pathway for both normoxic and hypoxic conditions. Adapted from: <http://being-bioreactive.com/2014/06/27/focus-on-the-hypoxia-pathway/>

Both subunits are also distinctive namely by their location, degree of induction controlled by O_2 concentration and functionality. While HIF-2 α expression appears to be induced by extremely low levels of oxygen and triggered exclusively in endothelial cells and vascularized tissues, HIF-1 α is thought to be expressed in all types of cells in mammalian at higher levels of oxygen concentration [113,117].

5.3 The impact of low physiological oxygen tensions

Hypoxia has been widely used in the past few years for research purposes in cell culture. However, the way it affects cell behavior is not yet fully understood or can be even quite controversial. These cultures in low O_2 environments have been studied for a long period of time, since 1972 when Allan Richter and his colleagues discovered that culturing cells in hypoxic conditions could help improving the plating efficiency by increasing the amount of cells that adhered to a plastic dish [118].

Oxygen concentrations of 1 % have been shown to be promoters of the osteogenic differentiation in bone marrow mesenchymal stem cells [119]. Pre-culturing human ASCs at 5% O_2 concentrations promotes the great ability of these cells to differentiate towards the chondrogenic lineages [120]. Hypoxic conditions of 2% O_2 increases also the potential to differentiate into the adipogenic and osteogenic lineages on the same type of cells [121]. Moreover, regarding the stemness and proliferation in the same severe hypoxic conditions, human ASCs were shown to have increased rates at low oxygen levels in comparison with the

normoxic. Nevertheless, regarding differentiation, these stress oxygen conditions reduced the capacity of ASCs to differentiate into the adipogenic and osteogenic lineages enhancing the chondrogenic one [122]. Hypoxia pre-conditioning enhanced the survival of hASCs and influenced the behavior of endothelial cells in vitro [123] besides increasing the wound-healing potential of ASCs [124].

Several researchers have also studied the angiogenic potential of hASCs in hypoxic conditions. Severe hypoxia increased the angiogenic paracrine activity of hASCs by increasing the production of VEGF-A and ANG [125]. Long-term hypoxia has been shown to improve the pro-angiogenic potential of hASCs [126]. The addition of growth factors to the culture of stromal cells derived from the adipose tissue is thought to produce the same effect [127]. Nevertheless, there are only a few studies regarding the effects of hypoxia in the whole SVF extract.

MATERIALS AND METHODS

1. CULTURE OF SVF CELLS

1.1 *Isolation of human adipose stromal cells*

In collaboration with Hospital da Prelada, Porto (supplier of lipoaspirate tissues).

Human adipose stromal cells were isolated and collected from subcutaneous fat tissue obtained through liposuction after informed consent of the patients (n = 3), in the scope of an agreement with Hospital da Prelada, Porto, after approval by the ethical committees of both institutions.

- Firstly, adipose tissue, processed until 24h after collection, was washed extensively with a great amount of a phosphate-buffered saline solution (PBS) (Sigma Aldrich, USA), and two phases were clearly noticed: an adipose fraction on the top and an aqueous one containing blood, which deposited on the bottom and was removed, by pipetting.
- Tissue digestion was then induced by incubating the adipose fraction (1:1) with a solution of PBS containing 0.05% of collagenase type II (Sigma Aldrich, USA) at 37°C for at least 30 minutes and up to 45 minutes, under agitation, until the tissue was sufficiently loose.
- Digested tissue was cleaned of any remnants of connective tissue and blood vessels by percolating it with a nylon mesh strainer with the assistance of forceps, followed by a centrifugation at 4 °C, 800 g for 10 minutes.
- After that period of time, supernatant was discarded and the obtained SVF was incubated with a red blood cells lysis buffer (154 mM of ammonium chloride, 10 mM of potassium bicarbonate and 0.1 mM of ethylenediaminetetraacetic acid – all from Sigma Aldrich, USA – in distilled water) for 10 minutes at room temperature to lyse undesirable erythrocytes.
- In order to restore the osmotic equilibrium, α – MEM medium (Life Technologies, United Kingdom) supplemented with 10% FBS (Life Technologies, United Kingdom) and 1% antibiotic/antimycotic (Life Technologies, United Kingdom) was added and the obtained mixture was centrifuged for 5 minutes at room temperature at 300 g.
- Lastly, red blood cells-free SVF was resuspended in α – MEM medium (Life Technologies, United Kingdom) supplemented with 10% FBS (Life Technologies, United Kingdom) and 1% antibiotic/antimycotic (Life Technologies, United Kingdom) and filtered by a 100 μ m nylon mesh cell strainer (Life Sciences, USA).

- To perform an accurate cell counting distinguishing nucleated cells from any remaining erythrocytes and debris, 20 µl of cell suspension were incubated with 180 µl of a

solution containing 3% acetic acid (VWR, United Kingdom) in methylene blue 0.05 wt. % in H₂O (Sigma Aldrich, USA). Finally, 2×10^5 cells were distributed for each well of 15.6 mm diameter 24-well culture sterile plates.

1.2 Conditions of culture

- Conditions of 21% (normoxia) and 5% of oxygen (hypoxia) were provided to the cultures. Low oxygen conditions were induced in a hypoxia modular incubator chamber (MIC-101, Billups-Rothenberg, USA) which was flushed for 6 minutes with a mixture of 5% O₂, 5% CO₂ and 90% N₂, according to manufacturer's instructions every day zero, one and five of culture.
- The chamber was then placed in an incubator (Galaxy CO-170S, New Brunswick) at 37°C, 5% CO₂ and cells were cultured up to 8 days.
- After 5 and 8 days of culture, conditioned medium (for ELISA, matrigel and scratch assays) and cells were collected for posterior characterization.

For matrigel and scratch assays, human dermal microvascular endothelial cells (HDMECs) and hASCs (passage 1-3) were used. A homogeneous culture of hASCs was obtained after the first passage of cells from the SVF since the remaining populations that it contains are lost during the culture process. The process by which cells are detached from the culture surface, changing their passage, happens when they reach approximately 90% of their confluence (percentage of space occupied by cells in the culture surface).

- Briefly, after removing the culture medium cells were washed with PBS and then incubated with tryPLE™ Express (Life Technologies, USA) at 37° C for 10 minutes in the humidified incubator chamber.
- After 10 minutes, cells detachment was confirmed on the microscope. The α -MEM medium was added and detached cells were centrifuged at 300g for 5 minutes.
- A pellet was obtained and hASCs were counted and reseeded or cryopreserved.

1.3 Cryopreservation

Cells cryopreservation is very useful for preserving cells at very low temperatures (-196° C) in liquid nitrogen. Enzymes and biomolecules remain in an inactive state for long periods of time

allowing cells to maintain a viable state and be subsequently thawed without losing its properties.

- Firstly, cells were detached from the well by incubation with trypLE™ Express X at 37° C for 10 minutes and then resuspended in α -MEM medium.
- After a centrifugation to obtain the pellet, cells were resuspended in a solution containing 10% of DMSO (Amresco, USA) in FBS (Life Technologies, United Kingdom) and distributed by cryovials (1 million of cells per each).
- The following step, the freezing, was processed slowly to avoid formation of ice crystals: first cells were placed at -20 ° C, then at -80 ° C and finally in liquid nitrogen at -196° C.

2. IN VITRO CHARACTERIZATION TESTS

2.1 Flow Cytometry

Flow cytometry is a technique used to perform measurements and/or analyze multiple characteristics of cells or particles (cell types in heterogeneous populations, cell surface expression and intracellular molecules, *inter alia*), as they pass through a laser beam. At that time, light will be scattered and then detected in the form of forward scatter (FS) or side scatter (SS) by appropriate detectors for the purpose. Forward scatter is cell size dependent while side scatter correlates with cellular content. In order to underline, through staining, certain characteristics of the cells, fluorochromes are commonly used. Each fluorochrome, which is connected to an antibody, retains its own emission wavelength, which in turn is going to be excited by the laser with the matching excitation wavelength. Then, by using a wide group of filters and mirrors is possible to steer the light (FS and SS) into defined wavelengths which are going to be captured by different detectors.

As stated by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy, one of the criteria that define the phenotype of mesenchymal stem cells is the

positive expression of surface markers CD105, CD73 and CD90, and lack expression of CD45, CD34, among other surface molecules. However, as a SVF is rich in various nucleated cells (adipocytes, blood cells, and others) it is slightly positive for the hematopoietic surface markers CD34, CD45, and CD31. Thus, after performing a gating strategy with DRAQ5 to discern only

nucleated cells in the SVF, tested markers were CD105 - FITC, CD73 - PE, CD90 - APC, CD45 - FITC, CD34 - PE and CD31 - APC (BD Biosciences, Germany).

Flow cytometry analysis were conducted at day zero, five and eight for each donor as it follows,

- 2 µl of each fluorochrome-conjugated antibody, mentioned previously, and 100 µl of sample (containing 1×10^5 cells) were added to each cytometer tube.
- Tubes were then vortexed and left for incubation in the dark during 20 minutes at room temperature.
- After that period of time, SVF cells and fluorochrome-conjugated antibodies were washed thoroughly with PBS and centrifuged at 350g, room temperature for 5 minutes.
- Deposited SVF cells were then resuspended in acquisition buffer, PBS with 1% of formaldehyde (Sigma, USA), and data was then acquired subsequently in a FACSCalibur flow cytometer (BD Biosciences, Belgium) and analyzed using Cyflogic version 1.2.1 (CyFlo Ltd, Finland).

2.2 DNA Quantification on lysed cells

The selected method to quantify DNA in lysed cells (in this case lysed by distilled water and frozen) involves the use of a fluorescent dye - PicoGreen – contained in a kit (Life Technologies, USA) according to manufacturer's instructions. When bound to dsDNA PicoGreen enhances its fluorescent intensity in accordance with the amount of DNA present in the sample. Thereby, it is often used to determine average dsDNA concentrations of a sample.

The principle of this method is to use a set of samples with known concentrations (standards) to enable an estimate (by comparison) of the fluorescence of the unknown concentrations. Therefore, knowing the concentrations of these standards and by reading their fluorescent intensity it is possible to obtain a calibration curve, from which, values of concentrations are going to be extrapolated for the samples. The procedure was executed as it follows:

- Standards with concentrations of 0, 0.0018, 0.023, 0.289 and 3,614 µg/ml were prepared by making serial dilutions of the λ DNA in TE 1x which was in turn diluted in ultra-pure water.
- To each well of a 96 well-plate, 100 µl of sample or standard plus 100 µl of the solution 1xTE plus Picogreen reagent were added. It is noteworthy that 3 replicates were made for each value of concentration for standards and for each condition of the samples.
- The fluorescent intensity was read at 485/528 nm using a microplate reader (SYNERGY HT, BIO-TEK, USA).

2.3 Matrigel Assay

The matrigel assay is a simple tube formation technique to evaluate the capability of vascular endothelial cells to assemble into capillary-like structures by embedding them in a basement membrane murine extract that mimics the extracellular matrix upon certain conditions.

- The formation of capillary-like structures was assessed by embedding human dermal microvascular endothelial cells (HDMECs) in a basement membrane extract, Matrigel (Corning, USA). This heterogeneous extract presents a liquid form at 4 °C while at temperatures between 16 and 37°C proteins polymerize solidifying it as a gel.
- The liquid Matrigel was casted into 96 sterile well-plates (32 µl per plate) for 30 minutes at 37°C, 5% CO₂ in a humidified incubator chamber.
- Immediately after, HDMECs at passage 2 in the respective conditioned mediums were seeded into each well in a density of 1.5 x10⁴/per well and were then placed in the incubator at 37°C for 24h.
- Cells were then stained with calcein-AM (Life Technologies, USA) and visualized under an Axio Observer inverted Microscope with incubation (Zeiss, Germany).

2.4 Scratch Assay

The scratch assay is a widely accepted method in the scientific community to evaluate cell migration rate in vitro. The principle of this technique involves the creation of a "acellular break" in a cell monolayer (through cell removal by scraping the central area of the bottom of the well or by placing a tissue culture insert, which blocks cell growth only in a central space flanked by cells).

- Recovered conditioned mediums for both conditions of biological samples used for in vivo testing were thawed and filtered with 0.22 µM strainers.

- Tissue culture inserts (ibidi, Germany) were attached to the middle of each well of 24 sterile well-plates and 70 µl of cell suspension containing $3,5 \times 10^4$ hASCs/well in α -MEM medium were added to each side of the insert.
- After 24h, cells were in confluent layer and, for that, the insert was removed and all α -MEM mediums were exchanged for the corresponding conditioned ones.
- Cell migration was monitored by capturing images in an Axio Observer Inverted Microscope with Incubation (Zeiss, Germany) every 5 minutes for 24 hours. The obtained images were acquired and processed with Zen 2012 software (Zeiss, Germany).

2.5 ELISA (Enzyme-Linked Immunosorbent Assay)

ELISA (Enzyme-linked Immunosorbent Assay) uses the sandwich principle (antibody-antigen-antibody binding) for colorimetric detection of specific proteins, hormones or other antigens. It can be very useful for measurements of antigen or antibody concentrations. To investigate the angiogenic potential of SVF CS the secretion of VEGF, FGF and PDGF-BB was quantified. Moreover, TIMP1 production was also quantified due to its essential role on promoting proliferation and anti-apoptotic functions in SVF CS. The assay was performed with mini ELISA Developments Kits (Peprotech, USA).

- Briefly, the surface of 96 sterile well-plates was coated overnight with a capture antibody followed by a 1h blockage of the unspecific bonds with BSA 1% (Sigma, USA).
- All steps in this test were interleaved with several washes.
- Conditioned mediums recovered from SVF cultures of all donors (n=3) were added to the wells (100 µl) for 2h.
- Antibody-antigen complex was then entrapped by a biotinylated antibody (2h of incubation) which in turn reacted with an avidin-horseradish peroxidase (avidin-hrp) conjugate that was allowed to attach biotin during 30 minutes.
- 100 µl of ABTS (Sigma, USA), the chromogenic substrate for the enzyme, which changes the intensity of the color in accordance with the amount of antigen present in the standard or sample, were added to the wells.
- The color development was then monitored every 5 minutes until 30 minutes in a microplate reader (SYNERGY HT, BioTek) and the absorbance was read at 405 nm with a wavelength correction at 650 nm.

2.6 Immunocytochemistry vs Immunohistochemistry

Immunocytochemistry and immunohistochemistry (see point 2.12) share the same principle but while the first one is exclusively performed on cells the last one is performed in tissues.

Both procedures are commonly used for detecting the expression of certain target proteins in cells or tissues by incubating them with a specific primary antibody, which will bind to it. Then,

by adding a secondary antibody specific to the primary it is possible to highlight the outcomes of interest.

2.7 Immunocytochemistry

The procedure was executed as it follows:

- Immediately after each time point, SVF cells were fixed in 10% formalin (Thermo Scientific, USA) for 30 minutes. After that, cells were washed with PBS.
- Firstly, cells were incubated with Triton X-100 (0.2 % solution in PBS) (Sigma, USA), a detergent capable of improving antibody's penetration, since the target protein is expressed intracellularly, for 10 minutes. Then, cells were washed with PBS (3 times).
- In order to block unspecific binding of antibodies, an incubation of 30 minutes with 3% BSA (Sigma, USA) in PBS was performed followed by an overnight incubation with the rabbit anti human-mouse CD31 (1:50) and mouse anti human CD146 (1:100) (Abcam, United Kingdom).
- After several washes and a one-hour incubation with secondary conjugated antibodies, donkey anti-rabbit 488 and donkey anti-mouse 594, 1:500, (Invitrogen, USA), cell's nuclei were counterstained with dapi (1:1000) (Sigma, USA) for 5 minutes and then analyzed in an Axio Imager Z1m fluorescence microscope (Zeiss, Germany).

2.8 Nuclear and cytoplasmic fractionation of the cells for Western Blot

HIF-1 α is oxygen responsive subunit. Under regular oxygen conditions, HIF- α subunits are hydroxylated being fastly degraded via the von Hippel-Lindau in the proteasome through ubiquitination. This degradation process occurs in the cytoplasm of the cells. At lower oxygen conditions HIF- α subunits translocate to the nucleus where they bind to the respective heterodimers. Both normoxic and hypoxic conditions are being studied being, thus, important to separate the cytoplasmic from the nuclear fraction.

All the extraction procedure was carried out as it follows:

- To delay the degradation process all the described steps were performed on ice or at 4°C.
- After each time point has been reached, the medium was removed and cells were washed in cold PBS. Then, 150 µl of a cytoplasmic buffer composed of 50 mM of tris

(Sigma, USA), 150 mM of sodium chloride (Panreac, Spain), 3 mM of magnesium chloride (Thermo Scientific, USA), 1mM of dithiothreitol (Sigma, USA), 1 mM of ethylenediaminetetraacetic acid (Sigma, USA), 1% of triton x100 (Sigma, USA) and protease inhibitor cocktail (important to delay the proteasome degradation) 1:100 (Sigma, USA), was added to the wells.

- The lysis buffer was incubated for 10 minutes and then, cells were centrifuged at a high velocity (13 000 rpm) for 20 minutes allowing nuclei to sediment and the recovery of the supernatant which contained the cytoplasmic fraction.
- The nuclear pellet was resuspended in 50 µl of a high saline buffer (10 mM of tris, 450 mM of sodium chloride, 1mM of dithiothreitol, 1 mM of ethylenediaminetetraacetic acid and protease inhibitor cocktail 1:100) and left for incubation during 30 minutes for disruption of the nuclear membrane. The disrupted nuclei were recovered by a second high-speed centrifugation and all the cell fractions were frozen at -80°C.

2.9 Total Protein Quantification (Bicinchoninic acid assay)

Loading the correct amount of protein for all the conditions in a western blot is extremely important for making viable comparisons and conclusions between them. For that, the total amount of protein present in a sample has to be quantified.

The total protein quantification assay uses bicinchoninic acid for colorimetric detection of the protein amount contained in a cell lysate. The principle of this assay is based on the formation of a purple-colored product that absorbs light as 562 nm. This purple water-soluble complex is formed by reacting bicinchoninic acid with a Cu⁺ ion by chelation.

- The quantification was performed using a bca protein assay kit (Fisher Scientific, USA).
- Firstly, the working reagent was prepared by mixing sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in sodium hydroxide with cupric sulfate (50:1).

- The reagent was left on ice and then BSA standards with concentrations ranging from 0 to 2000 µg/ml were prepared using both cytoplasmic and nuclear buffers. Right after, samples were thawed and diluted in the respective buffers in a 1:6 concentration.
- To each 25 µl of diluted sample or standard in replicates, 200 µl of working reagent were added per well of a 96 well-plate.
- The plate was then left for incubation at 37° C for 30 minutes and after that period of time and colour development the absorbance was read in a microplate reader (SYNERGY HT, BIO-TEK) at 562 nm.

2.10 Western Blot

Western Blot is a technique to identify proteins present in a cell or tissue extract by binding to specific antibodies. Although it comprises many tasks it's a fairly simple technique based on two main processes: electrophoresis and protein blotting. Electrophoresis occurs when an applied electric field moves charged particles towards the opposite electrode. This process is dependent on the size, structure and charge of the proteins. The proteins are then transferred to a nitrocellulose or polyvinylidene fluoride membrane to be stained with primary and secondary antibodies for detecting a specific antigen.

- Briefly, upon thawing and by using a 7.5% resolving gel (40% acrylamide (Bio-Rad, Netherlands), 1.5 M tris-HCl (Bio-Rad, Netherlands), 10% sodium dodecyl sulfate (SDS) (Sigma, USA), 10% ammonium persulfate (APS) (Bio-Rad, Netherlands), tetramethylethylenediamine (TEMED) (Bio-Rad, Netherlands) in distilled water), 50 µg of protein were loaded and then transferred into a nitrocellulose membrane (Bio-Rad, Netherlands).
- An overnight incubation with the primary antibody mouse anti-HIF1 alpha (BD Biosciences, USA) 1:500 was executed. Actin was used as a loading control in a concentration of 1:1000.
- After several 5 minutes washes, secondary horseradish peroxidase anti-mouse IgG (H+L) (Vector Laboratories, United Kingdom) was used in a 1h incubation.
- Finally, for detection, SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific, USA) was used following manufacturer's instructions and the membrane was scanned using a ChemiDoc XRS System (Bio-Rad, Netherlands) to

obtain chemiluminescent images with Quantity One 4.2.1 (Bio-Rad, Netherlands) software.

2.11 Implantation of SVF cell sheets following a hind limb ischemia model

All in vivo assays were performed after consent from the 3B's Research Group Animal Experimentation Ethical Committee.

The mouse hind limb ischemia model is a simple and viable attempt to address the problem of vascularization. Therefore, this model is a reproducible procedure that consists in the interruption of blood flow between two strategic points of the hind limb, through its sectioning for posterior implantation of cell-based devices in an attempt to reinforce or even restore the regular blood flux.

- Firstly, 6 months-old C57BL/6 male mice (Harlan, Netherlands) mice were anesthetized by continuous infusion of isoflurane (VIP300, Stoelting, Ireland) and then placed in the supine position in the operating table. Both hind limbs were shaved and cleaned with saline solution.
- The hind limb of the animal was attached in the operating table and the area around the skin surface was thoroughly disinfected with betadine. All the remaining steps were performed under the view of a stereomicroscope (Zeiss, Germany) for an accurate execution of the procedure.
- An incision of approximately 1 cm between the knee and the medial thigh was made and all the fat tissue surrounding the area was secluded.
- After separating the nerve, the femoral artery and vein were occluded using double knots at the ends of the vessels.
- The segment of femoral artery/vein between the distal and proximal knots was transected and the SVF cell sheets placed in the medial point between the occlusion points. All the four control animals didn't receive any cell sheet. Six animals were used per condition.
- After 33 days, animals were sacrificed and the explants were fixed for immunohistochemistry.

2.12 Immunohistochemistry

Fixed explants were processed in the spin tissue processor (STP120-2 Microm, United Kingdom) and embedded in paraffin (Thermo Scientific, USA) and cut (3.5 μ M – thick). The horse serum blocking, the Avidin Biotinylated Enzyme Complex and the secondary antibody

RTU biotinylated universal anti-rabbit were integrated in a VECTASTAIN Elite ABC kit while the components for the DAB solution were contained in a VECTASTAIN kit both from Vector Labs, USA.

- The slides were placed in the automatic stainer for deparaffinization.
- The slides were then heated near boiling in a microwave for antigen retrieval with a solution containing sodium citrate (10 mM sodium citrate, Fisher Scientific, USA; 0.05% tween, Bio-Rad, Netherlands) and then washed with distilled water.
- To block the activity of endogenous peroxidases and proteins, a 3% hydrogen peroxide (VWR, France) and protein blocking (horse serum) solutions were added to the slides for 20 minutes and 30 minutes respectively.
- After several washing procedures, rabbit primary antibodies to human CD 31 and α -SMA (Abcam, United Kingdom) were applied using antibody diluent solution (PBS + 5 drops of horse serum per ml) in the desired concentrations (1:50 and 1:100, respectively) at 4°C, overnight, in a chamber humidified with wet paper bed.
- The following steps were interleaved with washing procedures with PBS-tween. The secondary antibody RTU biotinylated universal anti-rabbit (mouse Ig, made in horse) was incubated for 20 minutes followed by the Avidin Biotinylated Enzyme Complex solution for more 20 minutes. Then, DAB solution (5 ml of H₂O + 2 drops buffer stock solution + 4 drops of DAB stock solution + 2 drops of hydrogen peroxide contained in the kit) was added to the slides following staining on the microscope.
- The slides were then left in water for 10 minutes and then dipped two times in Mayer's Hematoxylin. Finally, the slides washed again, dehydrated in ethanol 75%, ethanol 95%, ethanol 100% and xylene and mounted with Entellan® rapid mounting medium for microscopy (Merck Millipore, Germany).

2.13 In situ hybridization

In situ hybridization (ISH) is a technique that uses a labeled DNA or RNA probe to localize a specific sequence in a tissue. The binding of this probe to a sequence can be then detected by using a specific antibody.

The procedure was executed using a detection system core kit (Biogenex) according to manufacturer's instructions.

- Briefly, the tissue sections were deparaffinized and the nucleic acids retrieved by using a solution contained in the kit.
- After several washes, a fluorescein-labelled Alu II probe (Biogenex) specific to target Alu sequences of human DNA, was incubated in the slides overnight at 37°C.
- As a way to reduce the background, peroxidase and universal protein blocking solutions were then added before the incubation with the biotinylated mouse anti-fluorescein antibody for 30 minutes.
- In turn, the detection of this anti-fluorescein antibody was made with a second streptavidin-horseradish peroxidase conjugate antibody for 30 minutes. To produce a colorimetric reaction in the probe targets the substrate – DAB (Vector Labs, USA) – was incubated until adequate color development was achieved.
- Then, finally, the slides were dipped two times in Mayer's hematoxylin, washed extensively with water, de-hydrated, washed in xylene, and mounted using Entellan®.

2.14 Laser Doppler

The blood perfusion at the implantation site was assessed by Laser Doppler one hour after SVF cell sheets implantation and then each week up to 33 days. Blood flow in the hind limbs was measured with an Infrared Laser Doppler Imager (Moor Instruments, UK). Mice were anesthetized using a constant flux of isoflurane and placed in a supine position on a heated mat. After temperature stabilization, blood flow measurements were acquired. Ratios of the blood flow in non-affected limbs versus intervened limbs were used to assess blood flow recovery over time.

3. QUANTIFICATIONS

3.1 Quantification of capillary-like structures *in vitro*

Capillary-like structures were stained with the endothelial and hematopoietic marker CD31 and with CD146 for pericytes and screened with the transmitted and reflected light microscope Axio Imager Z1m (Zeiss, Germany). Parameters such as number of junctions, meshes, segments, branches and isolated segments were quantified in an angiogenesis plug-in designed for ImageJ 1.48[128]. Several parameters can be quantified depending on the purpose but we were focused on the number of nodes, junctions, meshes, segments, branches and isolated segments. A node is a pixel with at least 3 neighbors that when fused form junctions. The spaces between two junctions or one junction and one extremity are called segments and branches, respectively. When not branched, segments are considered as isolated while when enclosing a certain area

they form a mesh. An estimate of the parameters was obtained by choosing randomly 3 representative fields in each image and calculating an average for each.

3.2 Quantification of blood vessels *in vivo*

Perfused blood vessels, which present a brown color after performing the immunohistochemistry, were counted manually by an operator blind to the conditions. The wall of the vessels and the endothelial cells were highlighted by the antibodies α -SMA and CD 31, respectively, in different serial cuts.

4. STATISTICAL ANALYSIS

Data are herein expressed as mean + standard deviation for all conditions with three replicates for each. All statistical analysis was performed on GraphPad Prism 5 (GraphPad Software, USA). Flow cytometry and proliferation of SVF cells was analyzed using one-way ANOVA, Tukey's post-tests and Laser Doppler with two-way ANOVA, Bonferroni post-test since the obtained results followed a normal distribution. For non-parametric results obtained for ELISA and *in vitro* quantifications the analysis was performed with Kruskal-Wallis test.

Cells Sheets of Adipose Tissue Stromal Vascular Fraction as Vascularization Units for Tissue Engineering and Regenerative Medicine

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Abstract

One of the major concerns in Tissue Engineering (TE) and Regenerative Medicine (RM) strategies is the lack of a proper vasculature after transplantation that allows successful engraftment and survival of engineered tissues and organs. In this work, we describe a strategy to build angiogenic cell sheets (CS) by taking advantage of the stromal vascular fraction (SVF) of the adipose tissue. SVF cells were obtained by enzymatic digestion of subcutaneous lipoaspirate and cultured in sterile 24 well plates in normoxic ($pO_2 = 21\%$) and hypoxic ($pO_2 = 5\%$) conditions for up to 8 days in basal medium in the absence of extrinsic growth factors. The DNA quantification assay showed no significant proliferative differences between the conditions. Immunocytochemistry against CD 31 and CD 146 antibodies exposed spontaneous formation of a network of capillary-like structures, more developed for hypoxic conditions. ELISA assays revealed increased secretion of vascular endothelial growth factor (VEGF) in hypoxic conditions in comparison with normoxic. Confirming this, formation of capillary-like structures by endothelial cells cultured in Matrigel, using conditioned media recovered from the SVF cultures, was more prominent for hypoxic conditions. The same conditioned media were used in a scratch assay where the migration of human adipose stem cells was improved for hypoxic conditions. Histological analysis after in vivo implantation of 8 days SVF CS in a hind limb ischemia murine model revealed formation of new blood vessels near the implantation site. Moreover, Laser Doppler results demonstrated that the blood perfusion of the injured limb after 30 days was enhanced for the hypoxic CS group. Overall, SVF CS cultured in hypoxia shows promise and may represent a viable tool to promote functional vascularization in TE and RM strategies.

1. Introduction

Traditional strategies in TE and RM are mainly focused on the transplantation of single-cells or engineered tissues using 3D biodegradable matrices and cells into the injured site. The constant demand for effective therapeutic approaches led to the exploitation of the potentialities of stem/stromal cells as powerful tools for repairing lesions caused by defects, trauma or disease. Nevertheless, the balance between the risks and benefits of these cell-based approaches is not straightforward as they have limitations that often preclude the achievement of desired results. This comprises low anchorage of single cells at the site of interest[129], danger of exacerbated immunological responses to the implanted material[50] and the need for a well-orchestrated interplay of molecular factors demanded to create vascularization post-transplantation[130]. This process of vascularization is of extreme importance when considering engineered constructs since an abnormal supply of nutrients and oxygen often leads to their failure.

The revolutionary concept of Cell Sheet Engineering (CSE) arises in this context as an attempt to circumvent some of these issues. This scaffold-free approach allows the recovery of cells with its native extracellular matrix (ECM), produced throughout its culture, as a contiguous sheet that can be subsequently transplanted or act as a ramp for more complex constructs[94,97,131,132]. Besides this non-invasive cell harvesting, this technique avoids the cell loss in the transplantation site and is versatile enough to allow 3D manipulations by superimposing CS[52]. The potential of CSE has been extensively explored and it reached clinical studies stage in cornea[68], esophagus[133], myocardium[98], periodontium[66] and cartilage[100]. In our lab, we have proposed the use of CSE to engineer tissues such as skin[92] and bone[93]. In the case of a thick, complex tissue such as bone it is essential to address the issue of vascularization to achieve the fabrication of functional tissue. Therefore, diverse strategies have been designed, using not only endothelial, but also other types of cells to increase the vascularization of engineered constructs[134–136] such as the ones derived from adipose tissue[43,137,138].

Adipose tissue is regarded as a privileged source of mesenchymal progenitor cells due to its easy accessibility and abundancy[5]. This tissue hosts mature adipocytes as well as a stromal vascular fraction (SVF) which comprises several cell types including fibroblasts, pre-adipocytes, mesenchymal progenitors, endothelial and hematopoietic cells[40–42]. Due to this composition, the SVF of adipose tissue is regarded as highly angiogenic source [45,139,140]. Moreover, cells in the adipose tissue niche typically reside under low oxygen tensions (2%-

8%)[111] the so called “physiological normoxia”[101]. Several researchers hold this low oxygen pressures, natural in the stem-cell niche, as beneficial in many aspects such as maintenance of stemness in cells[122,141], promotion of cell proliferation[119,122,141], enhancement of the differentiation potential[142] and induction of several crucial genes for glycolysis (glucose transporters and glycolytic enzymes) and formation and maturation of the blood vessels such as VEGF, erythropoietin, angiopoietin and platelet-derived growth factor[113,117] Hypoxia-inducible factors (HIFs) are the family of transcription factors responsible for regulating the expression of those genes, controlling cellular responses, under low oxygen tensions.

Herein, we describe a strategy that brings together all of these concepts. CS made from SVF cells were cultured to hyperconfluence in normoxic and hypoxic conditions for up to 8 days in basal medium. Throughout the culture period, the development of capillary-like structures was visible in both conditions, being more prominent for cells cultured in hypoxia. Transplantation of SVF CS in mice, following a hind limb ischemia model resulted in enhanced restoration of blood flux for the condition of hypoxic implants.

2. Materials and Methods

2.1 Isolation of SVF cells

Human adipose stromal cells were isolated from subcutaneous fat tissue obtained through liposuction, from Hospital da Prelada (Porto, Portugal), after patients' (n=3) informed consent and under a collaboration protocol with 3B's Research Group, approved by the ethical committees of both institutions. Firstly, adipose tissue was washed extensively with PBS in order to remove the blood fraction. Tissue digestion was then induced with a solution of 0.05% of collagenase type II (Sigma, USA) in PBS (Sigma Aldrich, USA), at 37°C for 30 minutes, under agitation. Digested tissue was percolated through a nylon mesh strainer and was centrifuged at 4 °C, 800 g for 10 minutes. After that, the supernatant was discarded and the obtained SVF was incubated with a red blood cells lysis buffer (154 mM of ammonium chloride, 10 mM of potassium bicarbonate and 0,1 mM of ethylenediaminetetraacetic acid – all from Sigma, USA – in distilled water) for 10 minutes at room temperature. To restore osmotic equilibrium, SVF was washed in α – MEM medium (Life Technologies, United Kingdom) supplemented with 10% FBS (Life Technologies, United Kingdom) and 1% antibiotic/antimycotic (Life Technologies, United Kingdom) and filtered through a 100 μ m nylon mesh cell strainer (Life Sciences, USA). A final centrifugation yielded a pellet of red blood cells-free SVF and, in order to perform an accurate nucleated cell counting, 20 μ l of cell suspension were incubated with 180 μ l of a solution containing 3% acetic acid (VWR, United

Kingdom) and methylene blue 0.05 wt. % in H₂O (Sigma Aldrich, USA). Nuclei of nucleated cells were stained by methylene blue allowing a clear distinction of our cells of interest. Lastly, 2×10^5 nucleated cells were distributed by each 15.6 mm diameter-well of 24-well culture sterile plates.

2.2 Culture Conditions

Conditions of 21% (atmospheric) and 5% of oxygen were provided to the cultures. Cells were kept under low oxygen conditions using a hypoxia modular incubator chamber (MIC-101, Billups-Rothenberg, USA) which was flushed with a mixture of 5% O₂, 5% CO₂ and 90 % N₂, according to the manufacturer's instructions. The hypoxia modular incubator chamber and normoxic cells were placed in a humidified incubator at 37°C and 5% CO₂. Time points of 5 and 8 days were established for collection of the medium, which was aliquoted and frozen, as well as cells for posterior characterization.

2.3 Flow Cytometry Analysis

Flow cytometric characterization was performed as described by the *Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy* (ISCT)[37]. In this sense, fluorescent-labeled primary antibodies were used for the characterization of SVF comprised CD 105 - FITC, CD 73 - PE, CD 90 - APC, CD 45 - FITC, CD 34 - PE and CD 31 - APC (BD Biosciences, Germany). In addition, DRAQ5 (eBioscience, USA) was also used for nuclear staining, in order to discern the cells of interest from the any remaining erythrocytes and tissue debris (see supplemental figure 1). Flow cytometry was conducted right after isolation and after 5 and 8 days of culture for each biological sample. The referred antibodies were added to 1×10^5 cells, incubated according to manufacturer's instructions, washed with PBS and resuspended in PBS with 1% of formaldehyde (Sigma, USA). Nevertheless, only 2×10^4 cells were used for the analysis conducted in a BD FACSCalibur (BD Biosciences, Belgium) using the Cyflogic version 1.2.1 software (CyFlo Ltd, Finland).

2.4 DNA quantification on lysed cells

The selected method to quantify DNA in lysed cells (lysed by distilled water) involved the use of a Picogreen-based kit according to manufacturer's instructions (Life Technologies, USA) that detects double strain DNA by a quantitation assay. Briefly, standards were prepared by making serial dilutions of the λ DNA solution in TE 1x which was in turn diluted in ultra-pure water. To each 100 μ l of sample or standard, 100 μ l of the solution 1xTE plus Picogreen reagent were added per well. It is noteworthy that 3 replicates were made for each value of concentration for

standards and for each condition of the samples. The fluorescent intensity was read at 485/528 nm using a microplate reader (SYNERGY HT, BIO-TEK, USA).

2.5 Matrigel assay

Additional collected conditioned media from SVF cultures used in the in vitro tests were also thawed and filtered with 0.22 μ M strainers. The organization in capillary-like structures was assessed by seeding human dermal microvascular endothelial cells (HDMECs) (see protocol of isolation in the supplementary results) in Matrigel (Life Sciences, USA), a basement membrane extract. An amount of 32 μ l of Matrigel was added to each well of sterile 96 well plates in a humidified incubator for 30 minutes. HDMECs were then seeded on the top of the formed gel in a density of 1.3×10^4 cells/per well and the mixture was incubated for 24h in the humidified incubator of the microscope at 37° C, 5% of CO₂. After 24h HDMECs were stained with calcein (Life Technologies, USA) and micrographs were taken using an Axio Observer inverted Microscope with incubation (Zeiss, Germany).

2.6 In vitro scratch assay

Recovered conditioned media for both conditions of biological samples used for in vivo testing were thawed and filtered with 0.22 μ M strainers. Tissue culture inserts (ibidi, Germany) were attached to the middle of each well of 24 sterile well-plates and 70 μ l of cell suspension containing 3.5×10^4 hASCs/well in α -MEM medium were added to each well of the insert. After 24h, cells were in a confluent layer and the insert was removed. All α -MEM media were exchanged for the corresponding conditioned ones. Cell migration was monitored in an Axio Observer Inverted Microscope with Incubation (Zeiss, Germany) for 24h.

2.7 ELISA

The enzyme-linked immunosorbent assay was performed with mini ELISA Developments Kits (Peprotech, USA) following manufacturer's instructions. Briefly, the rabbit capture antibodies against VEGF, TIMP1, FGF and PDGF-BB were used to coat overnight the surface of 96 sterile well-plates followed by a 1h blockage of the unspecific bindings with BSA 1% (Sigma, USA). All steps in this test were interleaved with several washes. Conditioned media of all biological samples (n=3) were added to the wells (100 μ l) for at least 2h. To allow specific antigen binding, the antibody-antigen complex was entrapped by a biotinylated antibody (2h of incubation). After that, the latter reacted with an avidin-horseradish peroxidase (avidin-hrp) conjugate, which was allowed to attach biotin for 30 minutes. Then, 100 μ l of the chromogenic substrate for the enzyme, ABTS (Sigma, USA), were added. The color development was

monitored every 5 in a total of 30 minutes in a microplate reader (SYNERGY HT, BIO-TEK, USA) and the absorbance was read at 405 nm with a wavelength correction at 650 nm.

2.8 Immunocytochemistry

Immediately after reaching each time point, SVF cells were fixed in 10% formalin (Thermo Scientific, USA) for 30 minutes. To achieve cellular permeabilization, cells were incubated with Triton X-100 (0, 2 % solution in PBS) (Sigma, USA) for 10 minutes. Cells were washed with PBS (3 times) and then blocked for unspecific binding of antibodies with 3% BSA (Sigma, USA) in PBS. The primary antibodies rabbit anti human-mouse CD 31 (1:50) and mouse anti human CD 146 (1:100) (Abcam, United Kingdom) were then diluted to the desired concentrations and an overnight incubation with the cells was performed. After several washes and one-hour incubation with Alexa's secondary conjugated antibodies donkey anti-rabbit 488 and donkey anti-mouse 594 (Invitrogen, USA), cells were counterstained with DAPI (1:1000) (Sigma, USA) for 5 minutes and then analyzed in an Axio Imager Z1m fluorescence microscope (Zeiss, Germany).

2.9 Western Blot

All the described extraction procedure was carried out on ice or at 4°C. After each time point has been reached, cold PBS was added to the cells. After removing it, 150 µl of a cytoplasmic buffer composed of 50 mM of tris (Sigma, USA), 150 mM of sodium chloride (Panreac, Spain), 3 mM of magnesium chloride (Thermo Scientific, USA) , 1 mM of dithiothreitol (Sigma, USA), 1 mM of ethylenediaminetetraacetic acid (Sigma, USA), 1% of triton x100 (Sigma, USA) and protease inhibitor cocktail 1:100 (Sigma, USA), was added to the wells and allowed to lyse the cells during 10 minutes. Then, cells were centrifuged at 13 000 rpm for 20 minutes and the supernatant was recovered and stored at -80° C. The nuclear pellet was resuspended in 50 µl of a high saline buffer (10 mM of tris, 450 mM of sodium chloride, 1 mM of dithiothreitol, 1 mM of ethylenediaminetetraacetic acid and protease inhibitor cocktail 1:100) and left for incubation during 30 minutes for disruption of the nuclear membrane. A second high velocity centrifugation of 13 000 rpm was then performed and the nuclear extracts (supernatant) were left at -80°C. Upon thawing, total protein amount was quantified using a BCA protein assay kit (Fisher Scientific, USA) following manufacturer's instructions. By using a 7.5% resolving gel, 50 µg of protein were loaded and then transferred into a nitrocellulose membrane (Bio-Rad, Netherlands). Primary antibody mouse anti-HIF1 alpha (BD Biosciences, USA) was then diluted to the desired concentration of 1:500 and the membrane was incubated overnight with it. After several washing steps, the secondary antibody horseradish peroxidase anti-mouse IgG

(H+L) (Vector Laboratories, United Kingdom) 1:5000, was incubated with the membrane for 1h. For detection, SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific, USA) was used following manufacturer's instructions and the membrane was scanned, in order to create chemiluminescent images using a ChemiDoc XRS System (Bio-Rad, Netherlands) with Quantity One 4.2.1 (Bio-Rad, Netherlands) software.

2.10 *In vivo* tests

The transplantation of SVF CS from 8 days cultures in hypoxic and normoxic conditions was carried out following the establishment of hind limb ischemia in C57BL/6 (Harlan, Netherlands) male mice. Briefly, mice were anesthetized with a continuous flux of isoflurane (VIP300, Stoelting, Ireland). An incision of approximately 1cm was made starting on the knee towards the medial thigh and all the fat surrounding the area was seclused. After separating the nerve, the femoral artery and vein were occluded using double knots at the ends of the vessels. The segment of femoral artery/vein between the knots was transected and the CS placed between of the occlusion points. Six animals were used per SVF CS condition. The controls were made with four animals that didn't receive CS. After 1h, the animals' hind limbs were monitored with laser Doppler (LDI2-IR, Moor Instruments, United Kingdom) analysis and then every week until 33 days post-surgery, time that corresponds to the sacrifice of the animals and recovery and fixation of the explants for immunohistochemistry.

2.11 *Immunohistochemistry*

After fixation, explants from *in vivo* experiments were processed in the spin tissue processor (STP120-2, Microm, United Kingdom), embedded in paraffin (Thermo Scientific, USA) and cuts of 3.5 μ m were made. The sections were then dip in a sodium citrate solution (10 mM sodium citrate, Fisher Scientific, USA; 0.05% tween, Bio-Rad, Netherlands) and then heated near boiling for antigen retrieval. Non-specific peroxidase activity was inhibited during 20 minutes with a 3% hydrogen peroxide solution (VWR, France) and protein binding using horse serum contained in a VECTASTAIN Elite ABC kit (Vector Labs, USA) according to manufacturer's instructions. After several washes, rabbit primary antibodies to human CD 31 and α -SMA (Abcam, United Kingdom) were diluted to the desired concentrations of 1:50 and 1:100, respectively, and incubated with the samples at 4°C overnight, in a humidified chamber. Then, a secondary antibody RTU biotinylated universal anti-rabbit (mouse Ig, made in horse) was applied for 20 minutes before the incubation with an Avidin Biotinylated Enzyme Complex (ABC) (20 minutes), both solutions contained in the VECTASTAIN kit. A DAB solution (Vector Labs, USA) was then prepared following manufacturer's instructions and added to the

slides following color development on the microscope. The slides were washed, dipped two times in Mayer's Hematoxylin, washed again and then de-hydrated in ethanol 75%, 95%, and 100%, passed through xylene and mounted with Entellan® rapid mounting medium for microscopy (Merck Millipore, Germany).

2.12 *In situ* Hybridization

To detect cells of human origin in the explanted tissue, an *in situ* hybridization detection system core kit (Biogenex, USA) was used for detecting the human specific *Alu* repetitive DNA sequences, according to manufacturer's instructions. Briefly, the slides were deparaffinized and the nucleic acids retrieved using the appropriate solution of the kit. Then, a fluorescein probe (Biogenex, USA) specific to detect *Alu* sequences was incubated in the slides overnight at 37°C and blocking was made with peroxide and universal protein solutions. Immediately after, a secondary biotinylated mouse anti-fluorescein antibody was added to the slides for 30 minutes. Then, a second HRP conjugated antibody was added followed by its substrate, DAB (Vector Labs, USA), for color development. Lastly, the slides were dipped two times in mayer's hematoxylin, washed extensively with water, de-hydrated, washed in xylene, and mounted using Entellan®.

2.13 Quantification of capillary-like structures *in vitro*

Stained capillary-like structures after immunocytochemistry for CD 31 and CD 146 were screened with a fluorescence microscope (Imager Z1m, Zeiss, Germany). An angiogenesis plug-in was used to quantitatively analyze the formation and organization of pseudo capillary-like structures by endothelial cells in appropriate culture conditions for ImageJ 1.48[128]. Several parameters can be quantified depending on the purpose but we were focused on the number of nodes, junctions, meshes, segments, branches and isolated segments. A node is a pixel with at least 3 neighbors that when fused form junctions. The spaces between two junctions or one junction and one extremity are called segments and branches, respectively. When not branched segments are considered as isolated while when enclosing a certain area they form a mesh. By choosing 3 random representative fields in each image and averaging the values, all the parameters were estimated.

2.14 Quantification of blood vessels *in vivo*

Neo blood vessels, which presented a brown color after performing immunohistochemistry against human CD 31 and smooth muscle actin, were counted manually over a maximum depth of 300 µm from the implantation site by an operator blind to the conditions.

2.15 Statistical Analysis

Data are herein expressed as mean + standard deviation for all conditions with three replicates for each. All statistical analysis was performed on GraphPad Prism 5: for results with a normal distribution ANOVA was executed while for non-parametric ones Kruskal-Wallis test was used.

3. Results

3.1 Flow Cytometry

A gating strategy was designed by incubating cells with DRAQ 5 (supplemental figure 1), which allowed a clear distinction between nucleated cells (our cells of interest) and undesired erythrocytes and debris. SVF cells were analyzed immediately after isolation and after 5 and 8 days of culture for both conditions. Right after isolation, the obtained cell population is quite heterogeneous displaying a higher percentage of cells positive for CD 34 and CD 31 (51.91 ± 21.20 and 52.38 ± 26.14 , respectively), comparatively to the ones exhibiting the well-established mesenchymal markers CD 105, CD 73 and CD 90 (30.68 ± 21.55 , 37.81 ± 31.04 and 81.43 ± 20.69 , respectively). For 5 and 8 days of culture, the percentage of harvested SVF cells positive for mesenchymal progenitor cell markers increased substantially when compared to freshly-isolated cells (0 days), with almost the total amount of cells exhibiting a mesenchymal stem cell marker pattern. For the hematopoietic and endothelial markers (CD 34 and CD 31) the opposite can be noticed, with the percentage of cells expressing these markers decreasing over time (see figure 1). However, no significant differences were found between the hypoxic and normoxic conditions.

3.2 DNA quantification on lysed cells

Proliferation of SVF cells under conditions of normoxia and hypoxia was evaluated through dsDNA quantification. Normoxic cells show marginally higher DNA content after 5 and 8 days of culture. However no statistical differences were found between the conditions. Also, the number of cells for 5 and 8 days is quite similar for both conditions.

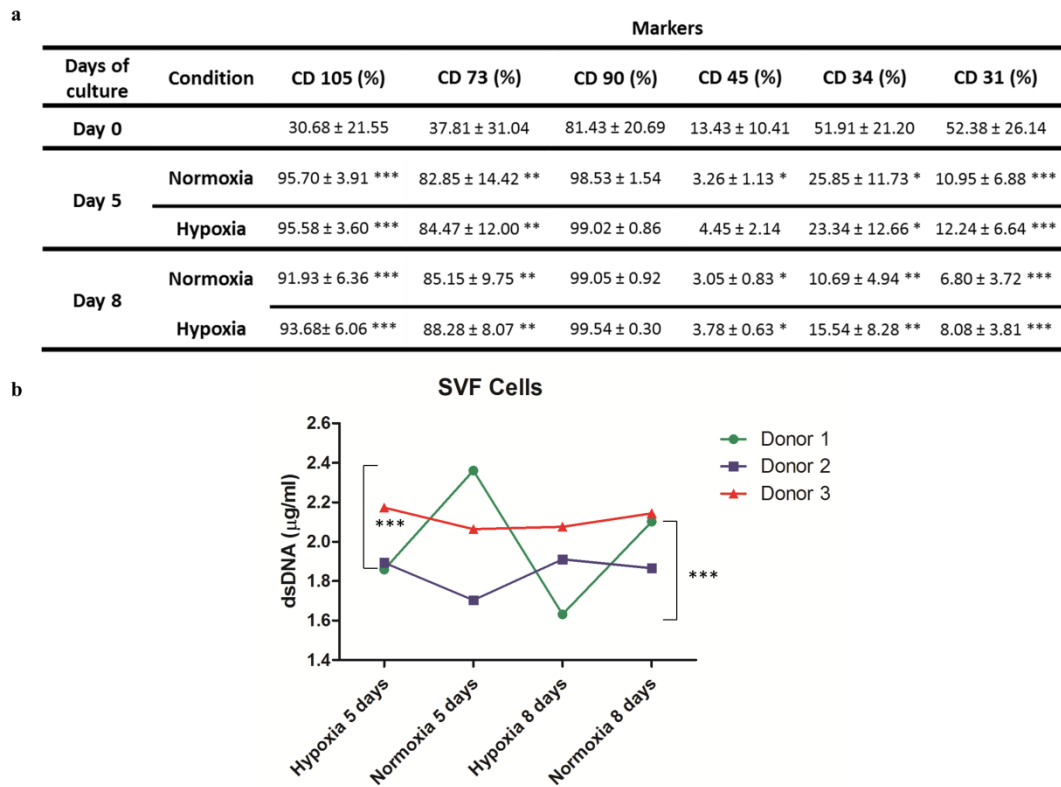


Figure 1 – a. Flow cytometry results obtained after using a gating strategy with DRAQ5 right after isolation (0 days) and for the time points 5 and 8 days. Mesenchymal (CD 105, CD 90, CD 73) and endothelial (CD 31) and hematopoietic (CD 45, CD 34) markers were used. b. DNA quantification on lysed SVF cells for 5 and 8 days in hypoxic and normoxic conditions. DNA results showed significant differences for a p-value < 0.05 for donor 1 between hypoxic and normoxic conditions both at 5 (***) and 8 days (**). Statistically, both flow cytometry and DNA quantification were analyzed using one-way ANOVA and Tukey's post-tests.

3.3 Matrigel Assay

Angiogenic potential of media conditioned by SVF cells was assessed in a matrigel assay using HDMECS (see figure 2). The results show that stimulation of tube formation was greater for media collected from hypoxic samples, with network development and interconnection comparable to the ones in the angiogenic growth medium condition. Moreover, this development and interconnection seems to be more evident for both conditions at 5 days.

3.4 Scratch Assay

For assessment of the cell migration rate in vitro a scratch assay was performed with hASCs for measuring the ability of these cells to migrate towards an “acellular break” when incubated with media from the cultures. The rates of hASCs break closure are more evident at 5 days for both normoxia and hypoxia when comparing to the use of fresh medium and at 8 days for the hypoxic conditions.

3.5 ELISA (Enzyme-linked Immunosorbent Assay)

The results displayed in figure 2 show that the secretion of all growth factors tested in the whole set of conditions increased from day 5 to day 8. One major angiogenic factor, VEGF, is slightly over-secreted for all samples in hypoxic conditions. However, no significant differences were found within each biological sample between the normoxic and hypoxic conditions for the analyzed growth factors after statistical analysis.

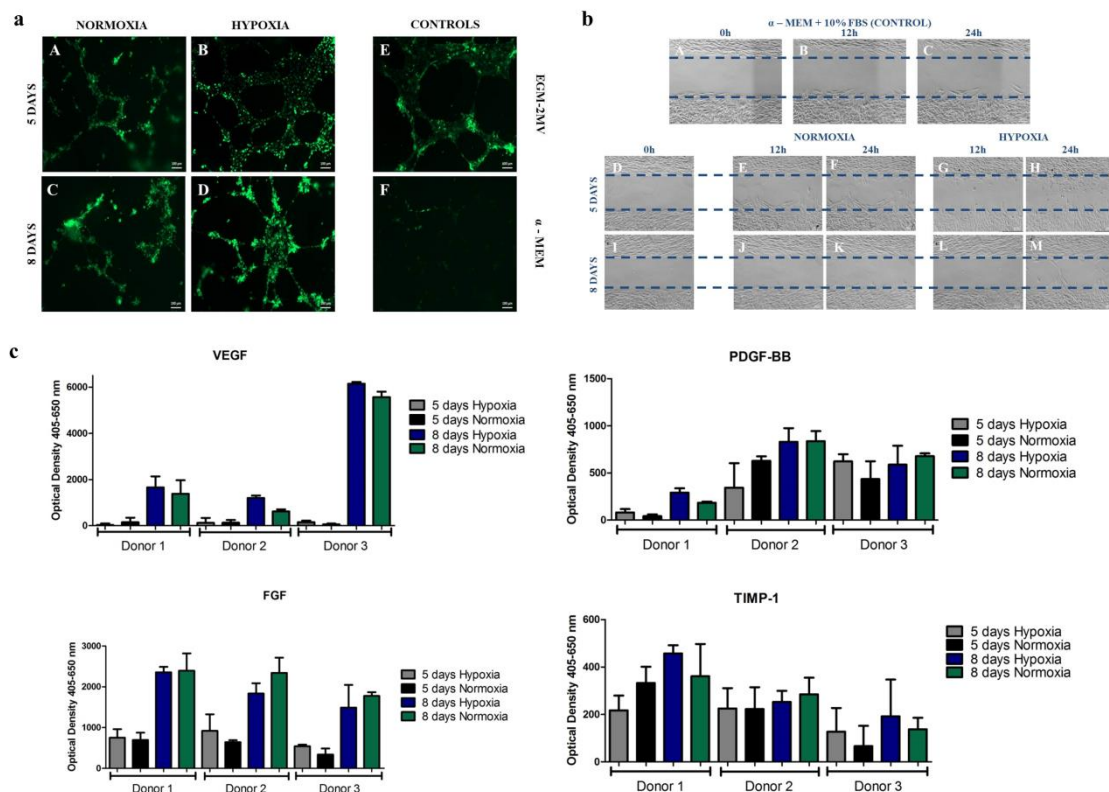


Figure 2 – a. Matrigel assay of HDMECs stained with Calcein-AM using conditioned media withdrawn from donor 3 SVF cell culture in normoxic condition after 5 A) and 8 days C); and from hypoxic cultures after 5 B) and 8 days D). Controls were made with VEGF-containing endothelial cell growth medium which, as expected, stimulated the in vitro formation of capillary-like structures for HDMECS E) and with α -MEM that didn't promote the same. b. Scratch assay performed with conditioned media from normoxic and hypoxic SVF cultures. Controls were made with α -MEM supplemented with 10% FBS at the beginning of the experiment a) and after 12h and 24h b) and c). Cell migration was assessed at 0h D) and I), 12h and 24h for media collected from 5 days normoxia D), E), F) and hypoxia G), H) samples and 8 days normoxia J), K) and hypoxia L) and M) samples. c. Quantification of growth factors release to the medium by SVF cells assessed by ELISA, including vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF-BB), fibroblast growth factor (FGF) and tissue inhibitor of metalloproteinases (TIMP1). Statistical analysis was performed using Kruskal-Wallis test and Dunn's Multiple Comparison post-test.

3.6 Immunocytochemistry

Performing immunocytochemistry against CD 31 and CD 146 assessed endothelial tubular structures formation by self-assembly of SVF cells. Endothelial cells (displayed in the images in green and red), together with pericytes (only red) are organized in a complex and interconnected capillary-like network. The structures are more developed and prominent for cells in hypoxia after 5 and 8 days of culture, than in normoxia culture conditions. The difference between the two conditions is more evident for donor 3, which presented the higher values for branching and segments, nodes, junctions and meshes development. On the contrary the number of isolated segments decreased. For this sample, higher values for nodes and junctions for 5 days hypoxic cells correlate well with the round-shaped morphology of the structures. In normoxic conditions, the low development of structures at 5 days is evidenced by the negligible values for segments and branches formation and the higher values for isolated segments. For the 8 days hypoxic cells the number of segments and meshes decreases although the values for the branching are almost the same, in comparison to the 5 days cells. This reflects a mature highly-branched and interconnected network with more elongated segments as depicted in the picture. The decreased number of isolated segments reinforces the hypothesis that tubes were not organized as single and separate structures but instead were communicating with each other. For normoxia, the development of capillary-like structures was clearly delayed in comparison to hypoxia, with elongated segments appearing branched only after 8 days, albeit in lower numbers.

3.7 Western Blot

Western Blot analysis for HIF-1 α was employed to analyze qualitatively the expression of this protein in hypoxic and normoxic conditions. As expected, the band of this ~120 kDa protein is more pronounced for hypoxic conditions in comparison with normoxia. HIF-1 α inhibitor and cobalt chloride II were used as negative and positive controls, respectively. While for the inhibitor the band is less evident, an incubation of the cells with a chemical inducer of hypoxia, cobalt chloride II result in a strong band, as evidenced in the displayed figure 3.

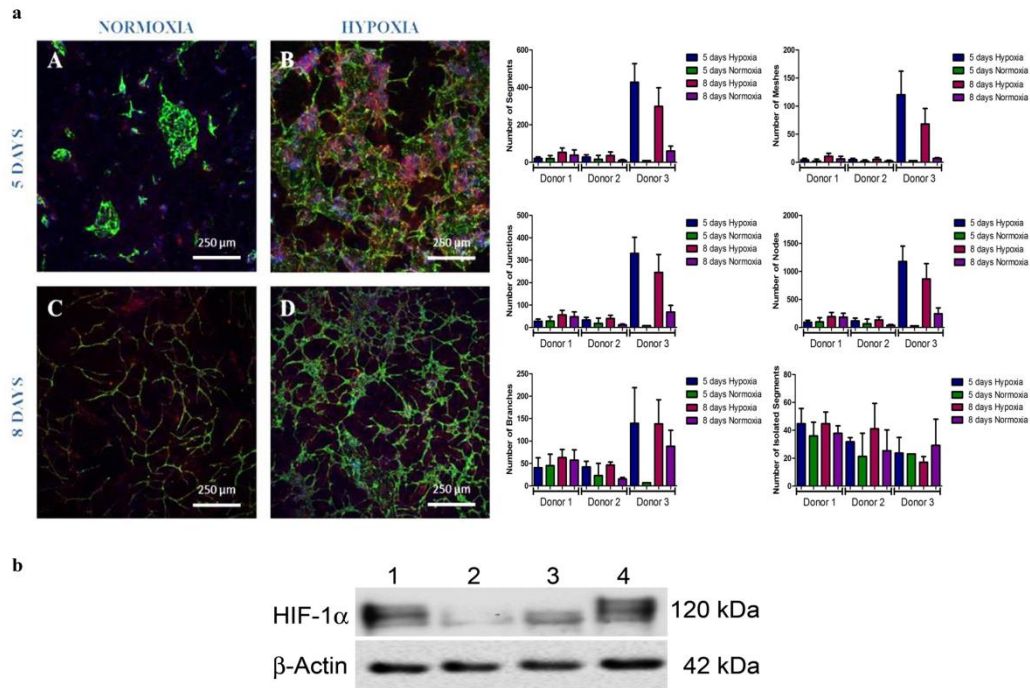


Figure 3 – a. Representative images after immunocytochemistry using endothelial marker CD 31 and CD 146 that stains pericytes in normoxic conditions after 5 A) and 8 days of culture, C) and in hypoxic after 5 B) and 8 days of culture D). Graphic representation of the quantification performed for all biological samples regarding the number of segments, isolated segments, meshes, branches, junctions and nodes, displayed as a mean \pm standard deviation. Statistical analysis was performed using Kruskal-Wallis test and Dunn's Multiple Comparison post-test. **b.** Western Blot analysis of the nuclear extracts of SVF cells for HIF-1 α and for the loading control, actin. Hypoxia is presented at 1), hypoxia supplemented with HIF-1 α inhibitor at 2), normoxia at 3) and cobalt chloride II supplementation at 4).

3.8 Immunohistochemistry

After 33 days, the animals were sacrificed and explants were performed. Immunohistochemistry results on the histological sections highlights neo-blood vessels formation (see figure 4). These neo-blood vessels, counted between the knots over a maximum depth of 300 μ m from the implantation site by an operator blind to the conditions, were present in higher numbers after hypoxic CS implantation. In one animal of each condition of donor 3, blood vessel regeneration could be macroscopically observed between the occlusion points (see supplemental figure 3).

3.9 *In situ* Hybridization

An *in situ* hybridization assay was performed with a specific *Alu* DNA probe to detect the presence of human cells in the explanted tissues. As evidenced by the figures, the stained nuclei in brown (due to DAB staining) correspond to human nuclei, enabling the assessment of their localization, namely near a blood vessel in the implantation site.

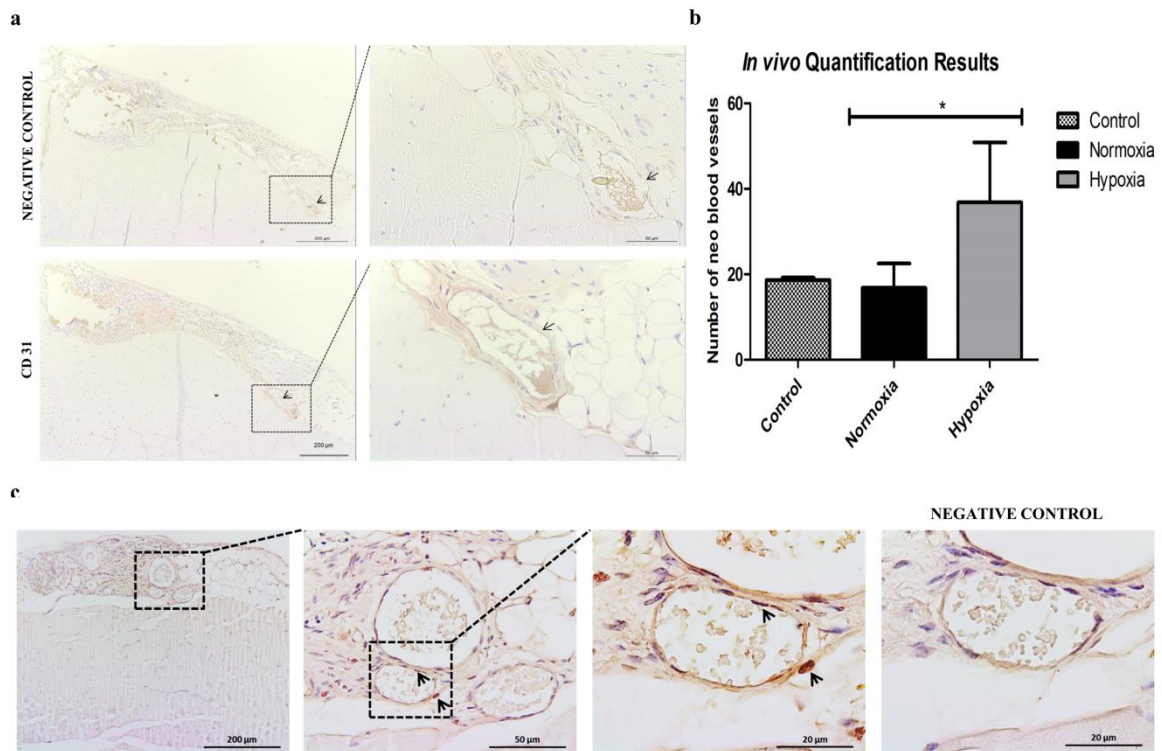


Figure 4 - SVF cell sheet implantation was performed following hind limb ischemia in a murine model. a. Representative images of histological sections after immunohistochemistry with CD 31 marker. Black arrows indicate the neo blood vessels. **b.** Quantification of neo blood vessels manually counted by an operator blind to the conditions and based on CD 31 positive cells. The graph presents a mean \pm standard deviation of all animals per condition. For statistical analysis one-way ANOVA and Tukey's post-tests were used. **c.** Detection of transplanted human cells as SVF CS by chromogenic *in situ* hybridization 33 days after implantation. Black arrows point to them. In purple are represented cellular nuclei.

3.10 Laser Doppler

Images obtained by laser Doppler (see figure 5 D, F) show that 33 days after the surgery there is improved blood flux when SVF CS were implanted. As confirmed by the complementary graph of the perfusion of the blood vessels this improvement is greater when a cell sheet derived from hypoxic cultures is implanted. However, there are no statistical differences between the two conditions.

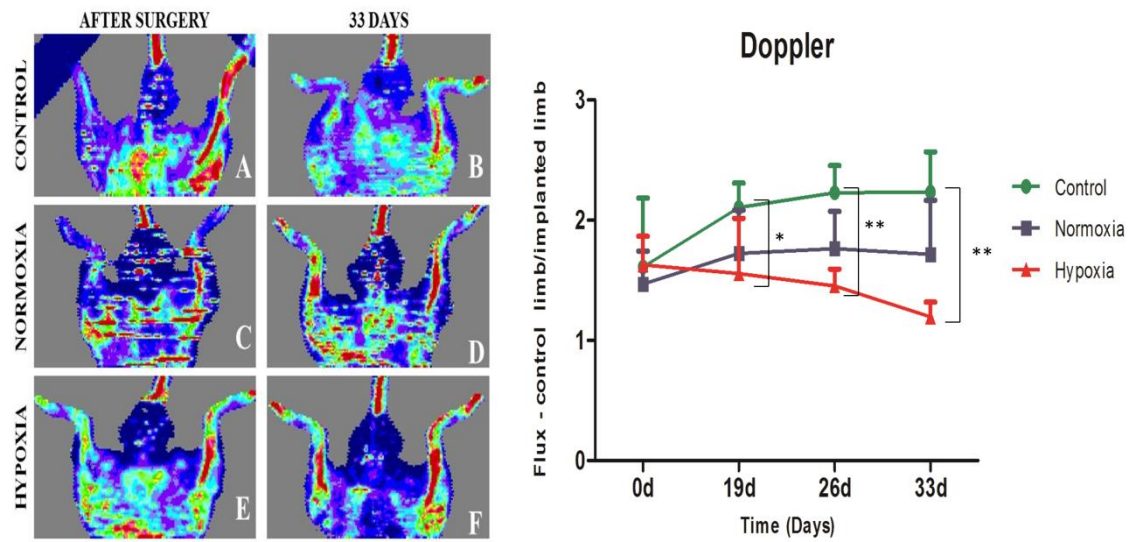


Figure 5 – Laser Doppler results collected up to 33 days. Blood perfusion assessment shows that the animals that received the hypoxic cell sheet had an enhanced blood flux after 33 days. However, statistical analysis shows no statistical differences between normoxic and hypoxic conditions but when comparing hypoxia with the control for a p-value < 0.05, difference was significant for 19 days (*), 26 days (**) and 33 days (**) with Kruskal-Wallis test, Dunn's post-test.

4. DISCUSSION

The successful engraftment of engineered constructs and subsequent regeneration of most tissues are highly dependent on their perfusion by blood vessels. Traditional strategies in TE and RM involve the culture of endothelial cells to develop *in vitro* pre-vascularized structures, which *in vivo*, through interaction with the host's vasculature could give rise to a functional and well-organized network of blood vessels. Nevertheless, the process of building pre-vascularized *in vitro* constructs is often compromised by its complexity, especially when dealing with thicker constructs and the lack of stability of the primitive plexus that can lead to its regression and/or poor functionality. In our group, we reported the use of endothelial cells isolated or in combined action with pericytes stacked between osteogenic CS for the formation of new bone[135,143]. Both works highlight the potential of pre-vascularizing a construct before implantation as these *in vitro* co-cultures resulted in enhanced formation of bone tissue when comparing to osteogenic CS implanted alone. These results encouraged us to develop a simple but extremely valuable neovascularization strategy stemming from the combination of three important concepts: cell sheet engineering, SVF culture and hypoxic conditioning. In this sense, stromal cells from the vascular fraction of the adipose tissue were cultured until hyperconfluence, forming a robust pre-vascularized cell sheet, implanted subsequently in ischemic mice.

Recently, scientists have been concentrating huge attention on the use of the stromal vascular fraction of adipose tissue for vascularization purposes, mainly due to its attractive composition: a heterogeneous cocktail of fibroblasts, pre-adipocytes, mesenchymal and endothelial progenitors and mural cells such as pericytes[40–42]. Mitchell et al. characterized phenotypically the cell populations derived from the enzymatic dissociation of the adipose tissue right after its isolation and along 5 passages[144]. For earliest stages they reported lower expression of the mesenchymal markers CD 105, CD 73 and CD 90 which increased overtime. Furthermore, the authors also noticed that SVF cells express the endothelial and hematopoietic markers CD 34 and CD 31, the first three times more than the latter. The same was reported by Miranville et. al, who divided the subpopulation CD 34+ in two subsets: the CD 34+/CD 31+ and CD 34+/CD 31-[138]. The isolation and culture of the CD 34+/ CD 31- subset resulted in an efficient differentiation of the cells towards the endothelial lineage and posterior revascularization *in vivo* when a hind limb ischemia murine assay was performed. In agreement with what was described before, CS derived from the SVF of adipose tissue reached the peak of expression of endothelial and hematopoietic markers after the isolation, decreasing their values until practically the most part of the population is positive for CD 105, CD 73 and CD 90 at 5 and 8 days. We hypothesized that the stromal populations characterized by the latter markers might grow faster than the subpopulations positive for both CD 34 and CD 31. Nevertheless, the expression of these markers is donor-dependent and a study with more patients would be

appropriate to reduce the variability and better understand the results regarding, in particular, the CD 34 and CD 31 markers. The presence of positive cells for the hematopoietic marker CD 45 decreased sharply with time, being considered even as negligible in light of what has been previously described[144].

For amplification of the angiogenic potential intrinsic to the SVF population, cells were submitted to continuous hypoxia, immediately after isolation. As suggested by several works in the literature, hypoxia triggers the HIF signaling pathway inducing biochemical and molecular changes that culminate with the expression of several genes associated with angiogenesis such as VEGF and PDGF-BB[113,114,117,145–147]. VEGF is the major responsible for the stimulation of neovascularization during vasculogenesis and angiogenesis and its expression is thought to be remarkably increased in low oxygen tensions. Secretion of angiogenic growth factors, assessed through ELISA, was demonstrated to be up-regulated for hypoxic conditions especially in the case of the VEGF. Matrigel and immunocytochemistry assays corroborate that hypoxic conditions stimulate cells capability to produce and release angiogenic growth factors due to the observed organization of cells in highly and interconnected capillary-like structures. Pericytes, detected by immunocytochemistry by CD 146 staining and absence of CD 31, might be crucial to the development and stabilization of the network of capillary-like structures throughout the 8 days of cell culture, as described previously in our lab[136]. In normoxic conditions, the presence of these tubes was also observed, although less robust. Koh et al. proposed that the formation of these structures might be due to a rearrangement of the endothelial and hematopoietic populations contained in the SVF[43]. Hence, we hypothesized that hypoxic conditions amplify this effect through the activation of the HIF signaling pathway which up-regulates angiogenic growth factors. Our western blot results, are consistent with the fact that hypoxic cultures are promoters of vasculature development and remodeling through HIF-1 α regulation[113]. As expected, incubation with an inhibitor of the HIF-1 α subunit resulted in poor accumulation in the nucleus as proved by the western blot assay and poor development of capillary-like structures in contrast to what is observed when cells are incubated with cobalt chloride, a chemical inducer of hypoxia (see supplemental figure 2). However, the specific role that each of the HIF subunits play in vascularization is not yet completely understood and more work needs to be developed in this sense.

Another test performed to assess growth factors production was a cell migration assay. This kind of assay represents an attempt to mimic the migration of cells in vivo in particular environments, as wound healing, e. g.. The enhanced rate of migration for media collected from normoxic and hypoxic conditions at 5 days when comparing to the use of fresh medium might have a straight relation with growth factors release. At 8 days the difference between the control and both conditions is not clear. Nevertheless, an ELISA assay for detecting growth factors

secretion to the media that may be influencing hASCs migration such as PDGF-AB, TGF- β 1, and TNF- α , which had been shown to possess chemoattractant activity, could be performed as future test[148].

Oxygen concentrations didn't seem to affect the proliferative rate of SVF cells throughout the culture period. The effect of hypoxia on the proliferation of adipose-derived stem cells in hypoxic conditions has been barely studied[149][119,122,141] and rarely in the case of SVF cells[150]. Further, as SVF cells achieved confluence before 8 days of culture, proliferation from 5 to 8 days is not robust. The degree of hypoxic induction is also an important factor influencing these results, i.e., as oxygen concentration is thought to vary between 2-8%[111] in the adipose tissue, therefore further studies using oxygen concentrations other than 5% need to be developed in order to take different conclusions.

In vitro results propelled us to explore the angiogenic potential of our pre-vascularized CS in a case of induced hind limb ischemia in mice. Transplanted CS showed that improved neovascularization occurred on the surrounding tissues close to the cell sheet implantation site, especially for the hypoxic CS. Moreover, *in vivo* results in one animal of each condition for donor 3 exhibited regeneration of the transected vessels. ELISA results show that the highest values of VEGF secretion as well as meshes, segments, branches, junctions and nodes after performing immunocytochemistry were obtained for this sample. Furthermore, the percentage of cells expressing the CD 34/CD 31 markers reached a peak at 8 days for cells isolated from it. We therefore hypothesized that all of these factors combined with the visible regeneration of the blood vessels displayed in the pictures (see supplemental figure 3) might be a result of a higher angiogenic potential of this sample when comparing to the others.

To our knowledge, the use of hypoxic SVF CS was never reported before but many other investigators have demonstrated the enhanced blood perfusion after transplantation of both SVF and human adipose-derived stem cells into ischemic sites[45,46,138,151]. This suggests that, clinically, hypoxic SVF CS might play promising active and protective roles against ischemia. Furthermore, Wittman et al. showed that the cryopreservation of SVF cells does not affect their viability nor the surface expression, adding to their clinical potential [137]. All of this potentiates the use of SVF CS as vascularization units for TE and RM applications.

5. CONCLUSIONS

The aim of the presented work was to develop highly angiogenic CS by culturing cells of the stromal vascular fraction (SVF) isolated from human adipose tissue in different oxygen conditions. Taken together, all results showed the viability of our strategy for this purpose as SVF cells, cultured in vitro as monolayered sheets, demonstrated high capacity of self-assembling in capillary-like structures, more notorious in hypoxic conditions. A western blot analysis highlighted the presence of the subunit HIF-1 α for both conditions with a more prominent band for hypoxic conditions. We therefore hypothesized that the enhanced angiogenic potential of the CS cultured in hypoxia might be correlated with the HIF pathway that results in the up-regulation of VEGF. Adding to our hypothesis, a matrigel assay showed that the tubular formations were more complex and interconnected when endothelial cells were cultured with hypoxic conditioned media. When implanted in mice, this SVF CS enhanced blood flow restoration and the formation of blood vessels after induced hind limb ischemia suggesting their suitability for use in clinical applications for neovascularization. This strategy shows the potential of hypoxic conditions and SVF CS for fabrication of highly angiogenic tools for TE and RM applications. Nevertheless, although being an extremely promising strategy, more studies need to be performed taking into account different variables: the concentrations of oxygen, the number of donors and the dynamics of all HIF subunits in vascularization. It could be also interesting to assess the interaction of SVF CS with CS built with cells derived from other different tissues by superimposing CS to engineer pre-vascularized grafts that could be then implanted to regenerate different kinds of tissues.

IV – ANNEX

SUPPLEMENTARY RESULTS

1. Gating Strategy performed for Flow Cytometry analysis

As SVF is a heterogeneous mixture of diverse cell types including non-nucleated and nucleated cells. DRAQ5 binds to dsDNA and allowed us to define a gate that included the nucleated cells of interest, during flow cytometry analysis. Gated cells were then analyzed for the different surface markers while non-nucleated cells and debris were rejected from the analysis.

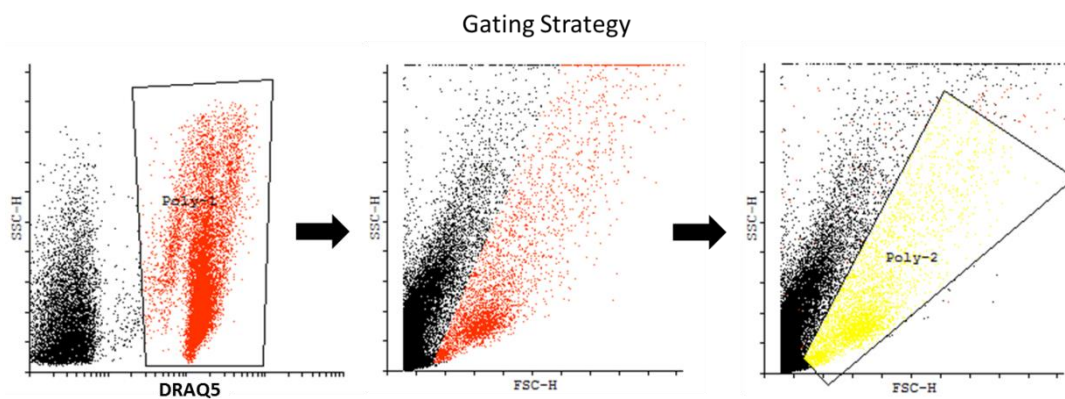


Figure 104 - Gating strategy for nucleated cells in the SVF population, by using DRAQ5. It allowed a clear distinction between our cells of interest and undesired debris.

2. Isolation of Human Dermal Microvascular Endothelial Cells

Skin Samples, with adjacent adipose tissue, were collected in sterile containers with 100mL solution of PBS (Sigma, USA) with 10% Antibiotic/Antimycotic (AB) (Invitrogen, USA), transported under controlled temperature conditions and processed within 24 hours after collection. Skin tissue was disposed from the adjacent adipose tissue, washed with PBS, cut in small fragments (roughly 1cm²) and incubated overnight in Dispase (2.4 U/mL) (BD Biosciences, USA) at 4°C. Dermis is a vascularized tissue therefore dermal microvascular endothelial cells (hDMECs) were isolated from the cellular suspension obtained prior to dermis enzymatic digestion with collagenase. In detail, the dispase solution in which dermis was mechanically dissociated was filtered through a 100 µm cell strainer and centrifuged for 5

minutes at 420 g. The pellet was then resuspended in Microvascular Endothelial Growth Medium (EGM-2 MV, Lonza, USA) and cells were plated in 0.7% gelatin (Sigma, USA) coated flasks. Endothelial cells exhibiting their characteristic cobblestone-like morphology and colonies arrangement were detected 72 hours after plating.

3. Supplementations with cobalt chloride II and HIF-1 α inhibitor

In order to confirm our results, chemical induction and inhibition of hypoxia effects were tested. Under regular oxygen conditions, HIF-1 α is hydroxylated on specific proline or asparagine residues by enzymes that demand dioxygen (O₂), Fe (II) and 2-oxoglutarate, through a catalytic process[112]. At oxygen stress conditions, this subunit translocates into the nucleus where it binds to its heterodimer, the HIF-1 β which is non-responsive to O₂ concentrations. A chemical inducer of hypoxia, cobalt chloride II, was used as a positive control once it mimics the hypoxic effect. The action of cobalt chloride is mainly inhibitory at the hydroxylation process level, by displacing Fe (II) from the catalytic reaction and inducing HIF-1 α accumulation. Also, an amidophenolic inhibitor of the HIF-1 α subunit was used. This inhibitor blocks the accumulation of cellular HIF-1 α in the nucleus, disrupting all the downstream effects of its accumulation.

3.1 Proliferation of SVF cells

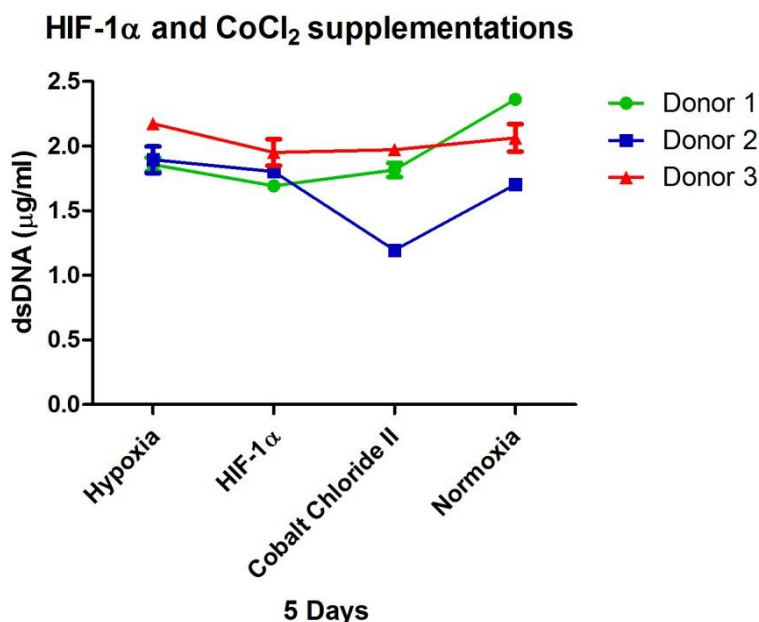


Figure 112 - Proliferation of SVF cells conditioned by the HIF-1 α inhibitor and cobalt chloride II assessed at day 5 of SVF culture. No statistical differences were found between the two conditions. However, the inhibitor and cobalt chloride II might be causing some cytotoxicity over the cells.

The results displayed on the Graph show that the proliferation rate was influenced by the supplementation compounds, with a decrease on the DNA content. However, no statistical differences were found when considering the concentrations of DNA for induced hypoxia and HIF-1 α inhibited condition. In addition, as cells were incubated from day 1 until day 5 with CoCl₂ they might be experiencing some cytotoxicity. Several studies report that the cytotoxicity of cobalt chloride II over the cells is time and dose dependent[152–156]. For lower concentrations and exposure time cobalt chloride did not induce significant cell death but it increased throughout the time of culture.

3.2 Immunocytochemistry

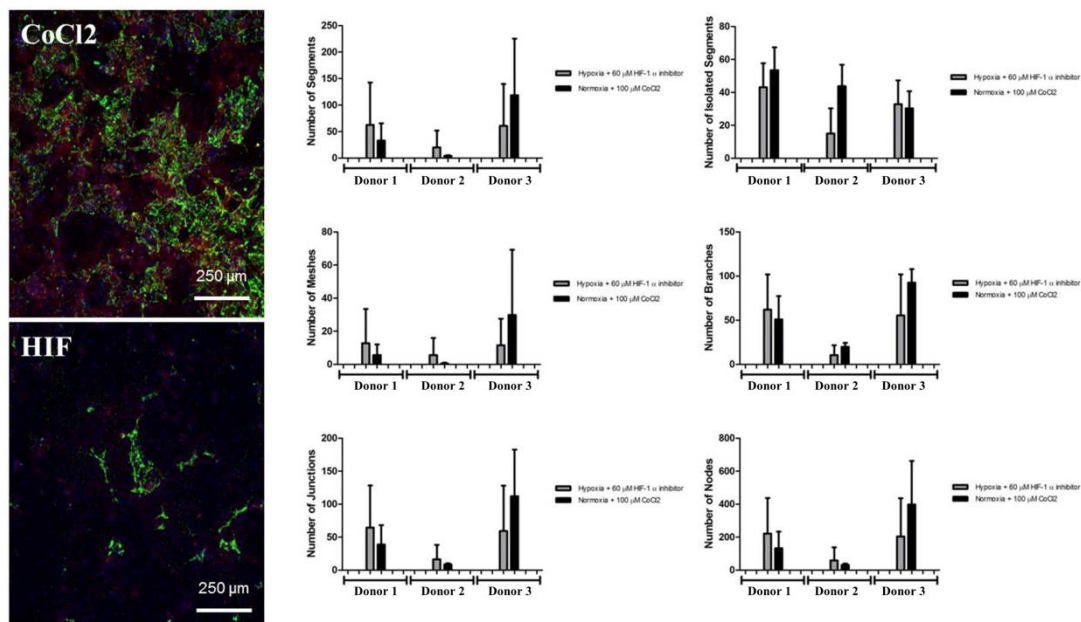


Figure 123 - Immunocytochemistry against CD 31 and CD146 and quantification of capillary-like structures assessing HIF-1 α inhibitor and cobalt chloride II effects over the cells. The lower (by HIF-1 α inhibition) and higher (through cobalt chloride supplementation) complexities may be associated with the HIF-1 α signalling pathway.

Although the structures were clearly more developed after the cobalt chloride supplementation, the quantification was not conclusive. Higher values in the counting for the cobalt chloride supplementation were expected as it mimics the hypoxic effect, but the poor quality of the images did not allow the proper use of the plug-in.

4. *In vivo* hind limb ischemia model



Figure 4 - In vivo tests were performed following a hind limb ischemia murine model. After 33 days, the animals were sacrificed and the explants fixed for immunohistochemistry. The image depicts the place of implantation after sacrifice. Inside the boxes are visible the regenerated vessels.

In vivo results of SVF cell sheet transplantation after inducing ischemia. One animal of each condition for donor 3 exhibited regeneration of the transected vessels. Elisa results show that the highest values of VEGF secretion as well as meshes, segments, branches, junctions and nodes after performing immunocytochemistry were obtained for this sample. Furthermore, the percentage of cells expressing the CD34/CD31 markers reached a peak at 8 days for cells isolated from it. We therefore hypothesized that the regenerated vessels displayed in the pictures might be a result of a higher angiogenic potential when comparing to the other samples.

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